JOURNAL OF DAIRY SCIENCE

DECEMBER, 1956

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Call for Papers for the 1957 Annual Meeting

Important News Items

Nineteen Articles Dealing with Original Research

The Plum Island Disease Laboratory

The Problem of Antibiotics in Milk

Mastitis — The Struggle for Understanding

Biochemical Aspects of Cheese Ripening

Index to the Thirty-ninth Volume of the Journal

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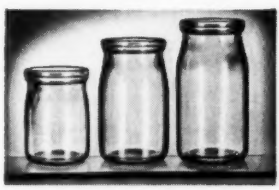

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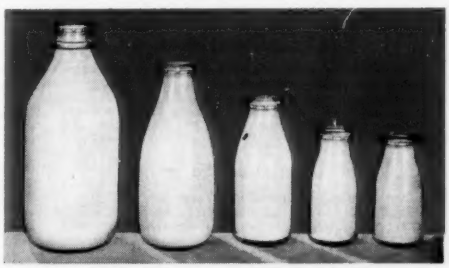

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Advertising

Advertising copy, insertion orders, contracts, and requests for information relating to advertising in the Journal should be addressed to the editor, P. H. Tracy, at the above address. Advertising plates should be sent directly to The Garrard Press, 119-123 West Park Ave., Champaign, Ill.

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Membership dues in the Association are $10.00 a year and should be sent to H. F. Judkins, Secretary-Treasurer, 32 Ridgeway Circle, White Plains, New York. Renewals should be made not later than December 1 each year.

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PEOPLE and EVENTS
in the Dairy Science World

Dairy Products Improvement Institute to Hold Meeting

The 10th annual meeting of the Dairy Products Improvement Institute will be held Feb. 14, 1957, in the Keystone Room of the Hotel Statler, New York City. The meeting will feature several talks on subjects of current interest and importance to regulatory officials and to industry representatives.

The first speaker of the main session in the afternoon will be W. D. TIEDEMAN, Univ. of Michigan, School of Public Health, Ann Arbor, who will discuss the "Economic Squeeze on Regulatory Agencies and Its Effect on the Future of Milk and Milk Product Sanitation." R. W. METZGER, director of quality control for the Dairymens League Coop. Assoc., Syracuse, N. Y., will review and discuss "Dairy Farm Inspection Forms." J. D. FAULKNER, chief of the Milk and Food Program, U. S. Public Health Service, Washington, D. C., will discuss "The Dating of Pasteurized Milk."

Also included in the program will be a report by A. F. RANNEY, chief of the Brucellosis and Tuberculosis Eradication Section, Agriculture Research Service, Washington, D. C., on the "Current Status of Brucellosis Testing Programs." This will be followed by a brief report from D. H. RACE, field director, Dairy Products Improvement Institute, Inc., Ithaca, N. Y., on the Institute's quality control program "Requirements for the Sanitary Production of Sweet Cream and Milk for Manufacturing."

Cole to Head Minnesota Department

C. L. COLE, for the past 6 years superintendent of the North Central School of Agriculture and Agricultural Experiment Station, is the new head of the Univ. of Minnesota Dairy Department. He succeeds J. B. FITCH, who is now head of the Dairy Department at the Univ. of Arizona.

Dr. Cole is a native of Michigan. For 7 years he was in charge of animal breeding at Michigan State Univ. and later was business manager of the Colby Dale Farms near Romeo, Mich. He went to the Univ. of Minnesota in 1950, where he recently received his Ph.D. degree.

DEAN PLOWMAN, who recently completed the requirements for his Ph.D. degree at the Univ. of Minnesota, has accepted a position in the Breeding, Feeding and Management section of the USDA at Beltsville, Md., as assistant to N. D. BAYLEY.

ARTHUR C. PETERSON, formerly a research assistant at the Univ. of Minnesota, has joined the research staff of Swanson's & Sons at Omaha, Neb.

Thanks from A.D.S.A.

The Association is indebted to The Borden Company for paying the A.D.S.A. membership dues of the coach and the winning team in judging dairy products at the Eastern States Exposition. This year's winning team was from the Univ. of Massachusetts, and it was coached by FRANK E. POTTER.

The Association also appreciates the action of the Chicago Dairy Technology Society in paying the student affiliate dues for 1957 for the high man in judging each product at the Regional Dairy Products Judging Contest held at the International Dairy Show in Chicago.

Herrmann Receives Government Award

USDA's L. F. HERRMANN has received an award presented by the Washington chapter of the American Marketing Assoc. for outstanding contributions to the field of marketing. Dr. Herrmann, an economist in the Agricultural Marketing Service, was recognized for his publication, "Regulations Affecting the Movement and Merchandising of Milk," Marketing Research Report No. 98. He received the award from Chapter president JOSEPH G. KNAPP at a luncheon in the Washington Hotel, Oct. 25.

Oregon State Dairy Technologist Joins Eastlack in Colombia

Dairy Society International (formerly known as DISI) announced completion of its staff for the joint U.S.-Colombian long-range dairy program as it flew ANTONIO DIAZ MORALES to Bogota last week. He will join JOSEPH O. EASTLACK, head of the DSI-ANALAC project, aimed at improving the quantity and quality of milk for the Colombian people.

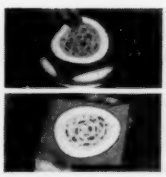
Diaz, a graduate of Oregon State Univ., attended the 20th Dairy Industries Exposition in Atlantic City, where he met many of the U. S. dairy industry leaders and also a number of the Colombian leaders with whom he will work. He also made a tour of dairy processing plants where he was briefed on the latest methods of handling dairy products. In Bogota, Diaz will act as technical advisor to Mr. Eastlack in this program sponsored by Foreign Agricultural Service of the USDA with DSI as cooperator.

There is no substitute for quality

How the *RAPID-FLO*® check-up for mastitis and sediment helps improve milk quality

It takes quality milk to produce quality dairy products—and quality begins on the farm.

Most producers take pride in their job—and do the job well with a reminder now and then of those factors so important in quality milk production. They're eager to avoid possible rejection—to earn top quality prices. The Rapid-Flo Check-Up for mastitis and sediment is an important aid in a quality farm milk program. Here's how it works:



1. After filtering each can of milk (10 gallons or less), the producer carefully removes the used filter disk from the strainer and places it on a cardboard to dry.

2. Examination of used filters will indicate constructive measures necessary to produce clean milk.

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Basically the program is aimed at raising the standards and increasing the use of dairy products by the Colombians, a task made difficult by the mountainous terrain and lack of transportation facilities. It is financed through funds set aside from the sale of some $175,000 worth of agricultural surplus products to Colombia (under U. S. Public Law 480 of the 83rd Congress), for market development for dairy products. Under the FAS-DSI-ANALAC Agreement ANALAC plans to raise double the amount available from P. L. 480, to provide a working fund of nearly a million pesos.

Foundation for this project was laid earlier by a DSI market survey team, headed by GORDON LAMONT of Darien, Conn., a Nestlé Company director; PETER OLSEN of the Olsen Publishing Co., and IVAN PARKIN of Pennsylvania State Univ. Their report, submitted to the Foreign Agricultural Service in May, formed the basis of the long-range cooperative plan launched by DSI and ANALAC in September.

Diaz, who received his early schooling in Peru, was recommended to DSI by the Oregon State Dairy Commission, which helped finance his trip to Washington for briefing for the post. Diaz received his Bachelor's degree in dairy manufacturing at Oregon State College and has been in charge of the by-products plant for Fairview Farms in Portland. Previously he had been assistant to the chief of publications, Dept. of Agriculture in Panama, where he wrote and edited for *Revista de Agricultura*.

Tabler Joins Staff at Beltsville

K. A. TABLER has joined the Biometrical Services of the Agricultural Research Center at Beltsville, Maryland. He was formerly employed as a cooperative agent of the Dairy Husbandry Research Branch assigned to the Department of Dairy Science at the Univ. of Illinois.

News from Ontario Agricultural College

A course for bulk milk tank operators held at Guelph Oct. 1-10, was so successful that plans have been made to hold a second such meeting in April, 1957.

Canadian delegates attending the 14th International Dairy Congress in Rome, Sept. 24-26, 1956, were as follows:

H. A. DERBY — Div. of Markets, Can. Dept. of Agriculture, Ottawa.

H. L. BERARD — Ecole de Laiterie Provinciale, St. Hyacinthe, P.Q.

D. ROSE — National Research Council of Canada, Ottawa.

W. H. SPROULE — Ontario Agricultural College, Guelph.

J. M. NESBITT — Univ. of Manitoba, Winnipeg.

C. K. JOHNS — Dairy Technology, Can. Dept. of Agriculture, Ottawa.

H. ROULEAU — Consumer Section, Marketing Service, Can. Dept. of Agriculture, Ottawa.

Ohio State News

The 24th Annual Dairy Technology Conference has been scheduled for Feb. 5-8, 1957. The program will include discussions in the fields of butter, field work, market milk and engineering, and ice cream and condensed milk. As usual, timely problems and new developments will be discussed by leading persons from the industry and the academic fields.

Sixteen persons from 16 different organizations attended the 2-week short course in market milk concluded Nov. 16. A similar short course will be conducted in ice cream starting on Jan. 7.

A panel discussion on "Dairy Plant CIP Systems" was the featured event of the November meetings of the Maumee Valley, Northeastern Ohio, Central Ohio, and Cincinnati Dairy Technology Societies. Members of the panel were L. H. MINOR, Technical Service, Wyandotte Chemical Co.; D. A. SEIBERLING, Dept. of Dairy Technology, O.S.U.; R. B. WATTS, State Sanitarian, Ohio Dept of Health; J. H. DRAYER, Deputy Health Commissioner, Ohio Dept. of Health; and E. B. BUCHANAN, Deputy City Health Commissioner of Cleveland.

Back Issues of Journal Needed

To take care of orders for back issues, the Association needs copies of the following numbers of the *Journal of Dairy Science:*

1918	3, 4, 5, 6
1919	all issues
1921	4, 5, 6
1922	1, 3, 6
1923	all issues
1924	4
1925	1, 5
1926	1, 2, 3
1928	1
1930	3
1931	5
1946	3, 4
1947	1, 2, 3, 4, 5, 12
1953	1, 2
1955	1, 3, 4, 5, 8
1956	2, 3

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Kentucky News

The Univ. of Kentucky's annual Dairy Student-Faculty Mixer was held Nov. 5. This annual dinner was attended by over 50 students and gave them a chance to become acquainted with the faculty of the Dairy Section. D. M. SEATH, head of the Section, introduced this year's Dairy Cattle Judging Team and announced the winners of the various trophies. The team placed first in the Southern Section, seventh at Waterloo, and first at the International in Chicago.

More than 115 dairy alumni and students attended the annual Homecoming Breakfast at the Univ. of Kentucky Dairy Section, Nov. 10. This annual event, sponsored by the Dairy Club, gives the students an opportunity to meet many outstanding leaders of the Kentucky dairy industry.

Mississippi Regional Champions

The Mississippi State dairy products team, coached by E. W. CUSTER, won the all-products cup at the regional judging contest held at Clemson College Oct. 26. The second place team was from Texas Tech. RONALD SAPPINGTON of the Univ. of Maryland, whose team placed third, was high individual score winner. There were eleven competing teams from southern and southwestern states.

Fike Joins Pennsalt Chemicals

J. E. FIKE has joined Pennsalt Chemicals Co. as a technical service engineer in the Chemical Specialties Division. In this capacity he will specialize in sales service on Pennsalt's line of B-K sanitation chemicals for the dairy and food processing industries.

Fike holds a B.S. degree from West Virginia Univ., where he was an instructor and assistant in dairy manufacturing, and an M.S. degree in agriculture from the Univ. of Maryland. He was quality control supervisor for Southern Dairies, Inc., in Norfolk, Va., prior to joining Pennsalt.

Dairy Scientist Receives Award at Exposition

OTTO F. HUNZIKER of La Grange, Ill., one of America's most noted dairy scientists, received the Dairy Industries Old Timers Club award for length of service in the field of dairy science at the 20th Dairy Industries Exposition Oct. 31 in Atlantic City. The award, an engraved gold medallion, was presented by MISS SHARI LEWIS of Daykin, Neb., America's dairy princess and official hostess of the Exposition. With Mrs. Hunziker, Dr. Hunziker is a regular visitor to the biennial expositions.

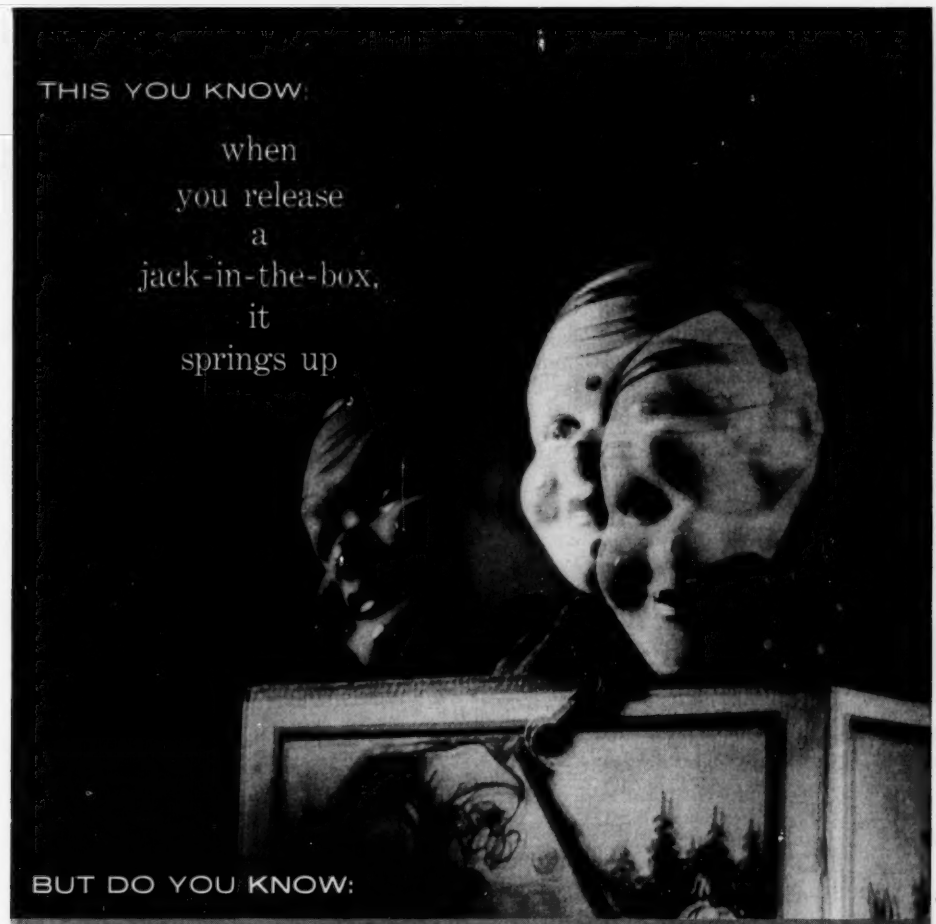
1955 Summary of Disease Outbreaks

C. C. DAUER and GRANVILLE SYLVESTER of the Morbidity Analysis Section of the National Office of Vital Statistics of the U. S. Public Health Service published in Public Health Reports, August, 1956, a "1955 Summary of Disease Outbreaks." A summary of the foodborne and waterborne disease outbreaks for the year will be of interest to Journal readers.

Foodborne and waterborne disease outbreaks reported in 1955 by vehicle of infection

Area	Water		Milk and milk products		Other foods	
	Outbreaks	Cases	Outbreaks	Cases	Outbreaks	Cases
United States	2	22	3	302	[1] 193	[1] 9,633
New England:						
Maine	—	—	–	—	2	127
Massachusetts	—	—	–	—	5	250
Middle Atlantic:						
New York	—	—	–	—	12	433
New Jersey	—	—	–	—	1	145
Pennsylvania	—	—	1	278	2	7
East North Central:						
Ohio	—	—	–	—	5	275
Indiana	—	—	–	—	3	89
Illinois	1	22	1	15	5	146
West North Central:						
Minnesota	—	—	–	—	3	180
Iowa	—	—	–	—	1	6
Missouri	—	—	–	—	3	120
South Datota	—	—	–	—	—	—
South Atlantic:						
Delaware	—	—	–	—	1	21
Maryland	—	—	–	—	2	355
Virginia	—	—	–	—	2	52
West Virginia	—	—	–	—	3	104
Georgia	—	—	1	9	—	—
Florida	—	—	–	—	1	181
East South Central:						
Tennessee	—	—	–	—	—	—
Mississippi	—	—	–	—	1	5
West South Central:						
Arkansas	—	—	–	—	5	178
Louisiana	—	—	–	—	3	54
Texas	—	—	–	—	2	78
Mountain:						
Idaho	—	—	–	—	4	17
Colorado	—	—	–	—	1	5
New Mexico	—	—	–	—	5	992
Utah	—	—	–	—	3	154
Pacific:						
Washington	—	—	–	—	5	31
Oregon	—	—	–	—	5	44
California	1	[2]	–	—	71	2,491
Hawaii	—	—	–	—	1	14
United States 1954	7	452	9	200	234	11,704
United States 1953	11	719	4	97	194	9,914

[1] Includes outbreaks among military personnel not listed in any State. [2] Not available.



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Completed Theses

M.S. Degree

ARTHUR J. NIELSEN — Changes in bacterial counts and flavor of dry milks, concentrated milk, and dry ice cream mix before and after recombination by various means and storage at low temperatures. Univ. of Minnesota.

VERNAL S. PACKARD — The influence of various packaging materials on the keeping quality of ice cream and on rates of temperature change during hardening and subsequent handling. Univ. of Minnesota.

Ph.D. Degree

VELVL W. GREENE — The effect of heat treatment of milk and milk powder on the development and activity of lactic acid cultures (cheese starters). Univ. of Minnesota.

WILLIAM G. MIZUNO — A study of the formation of acetoin by mixed lactic streptococci and leuconostoc. Univ. of Minnesota.

ARTHUR C. PETERSON — Some aspects of bacterial metabolism of unsaturated fatty acids. Univ. of Minnesota.

DEAN R. PLOWMAN — An evaluation of a breeding program and sires used in a selected dairy herd. Univ. of Minnesota.

IRA D. PORTERFIELD — The response of the mammary gland to the introduction of antigens. Univ. of Minnesota.

Boyd Joins Tennessee Faculty

L. J. BOYD recently joined the dairy faculty at the Univ. of Tennessee. A native of Kentucky, he received his B.S. and M.S. degrees from the Univ. of Kentucky. He received his Ph.D. degree from the Univ. of Illinois.

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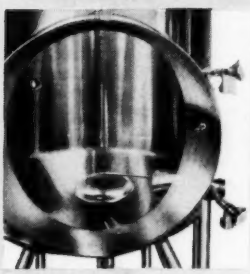

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Recent Deaths

JAMES M. SHERMAN, professor of bacteriology at Cornell since 1923 and head of the University's Department of Dairy Industry for 32 years, died Nov. 5. He was 66 years old.

Dr. Sherman relinquished his administrative duties as department head July 1, 1955, to devote full time to writing and research. His major research work concerned general biological studies of microorganisms; systematic studies of some of the important groups of bacteria, especially the streptococci; and investigations of the microorganisms of importance in milk, food products, soil, and industrial fermentations.

In 1948 he was cited for discoveries in the relationship between certain types of bacteria, which proved of great value in industrial processes and medical science. For his achievements North Carolina State College conferred on him the honorary degree of Doctor of Agriculture.

Dr. Sherman did his undergraduate work at the North Carolina school and received his B.S. degree in 1911. He was granted his M.S. degree in 1912 at the Univ. of Wisconsin and his Ph.D. degree from the same university in 1916. He was instructor and assistant professor at Pennsylvania State College from 1914 to 1917, and bacteriologist in the U. S. Department of Agriculture from 1917 to 1923.

At various times in his career he was a consultant to the U. S. Army Surgeon General and Chemical Corps; to the Federal Security Agency; to the U. S. Public Health Service; to the National Research Council; and to the State Department of Health. He was the U. S. delegate to the World Dairy Congress in 1931.

Dr. Sherman was the 25th president of the American Dairy Science Association and also served as president of the Society of American Bacteriologists. He is the author of more than 100 research papers in scientific publications, a book on laboratory methods in bacteriology, and a monograph on the streptococci. He served as editor-in-chief of the *Journal of Bacteriology* (1944-51), and associate editor of the *Bacteriological Reviews* (1937-1944). He was currently a member of the editorial committee of the *Annual Review of Microbiology*. For five years he was a member of the board of editors of the Cornell University Press and served for two terms as a member of the Publications Board of the Society of American Bacteriologists.

Dr. Sherman is survived by his widow, KATHERINE KEIPER SHERMAN; two sons, RICHARD H., a graduate student at the Univ. of Wisconsin, and THOMAS F., a Rhodes Scholar at Oxford Univ., England; and a daughter, MRS. JAMES H. LITTLEPAGE, Washington, D. C.

E. J. WEATHERBY, extension associate professor at the Univ. of Maryland, died November 5. Dr. Weatherby served as manager of the Artificial Breeders Cooperative, Inc., and has been responsible for creating a strong breeding program for the state of Maryland.

JOHN R. PERRY, assistant to the director of Plant Production, National Dairy Products Corp., who died Oct. 25, at 61, was one of the most widely known and respected men in the field of dairy technology. His activities were manifold and varied — covering Sealtest production functions, plant and equipment engineering, Health Department contacts, surveys of operations, attendance at government and industry meetings, and service on industry committees.

Among Mr. Perry's many industry activities were: the 3-A Sanitary Standards Committee of the Milk Industry Foundation; the Committee on Definitions and Standards of the IAMD; Advisory Committee, New Jersey Milk Control Board; and membership in several scientific associations. He was the author of a large number of magazine articles on various dairy subjects.

Mr. Perry served with the U. S. Army during World War I, enlisting as a private, and being commissioned as an officer in the Field Artillery. He was in combat on the Western Front, and his decorations included the Victory medal with three stars and the Verdun medal.

Mr. Perry was born near Bernardsville, N. J., and had lived in East Orange for 31 years, where he was active in community affairs. As a member of St. Bernard's Episcopal Church, Mr. Perry had served as Sunday School superintendent. He graduated from Rutgers College in 1920. On April 9, 1926, he received the rating of Professional Mechanical Engineer in New Jersey.

The case against "phantom" phosphorus

The evidence against "phantom" phosphorus — phosphorus that can't be utilized by farm animals — is well worth examining.

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STUDENT CHAPTER NEWS

A section devoted to the activities of dairy students

E. L. THOMAS, *Editor*

U. of C. Dairy Club Activities

The Blue and Gold Dairy Club on the Univ. of California's Davis campus recently launched its program for the current semester with a student-faculty Bar-B-Q. Plans have been made for a well-rounded program including prominent speakers, pertinent films, and social activities. Projects planned include compiling a directory of dairy industry alumni, students and staff; building a picnic-day float; and selling refreshments and complete sets of abstracts of papers presented to those attending the Dairy Industry Conference to be held at the Davis campus Jan. 28-30, 1957.

Idaho Dairy Students Receive Awards

The Virginia Dare Award at the Univ. of Idaho for 1956 was presented to Daryl Belts, a senior in dairy manufacturing. Jerry Fitch, selected as the outstanding dairy manufacturing student for the 1955-56 school year, was presented the consolation award of the Milk Industry Foundation and received the book "Legal Aspects of Milk Sanitation." The awards were presented at a regular meeting of the Idaho Dairy Science Club.

The Idaho Dairy Products and Dairy Cattle judging teams participated in the Pacific International at Portland, Ore., on Oct. 20. The Dairy Products team, coached by James Boyd, won the cheese trophy, and Emil Loe placed first in milk judging. The dairy cattle team, coached by K. R. Johnson, placed first in judging Jerseys with Tom Cooper being the high individual judge of the breed.

Massachusetts Dairy Club Sponsors Alumni Breakfast

On Oct. 13, the Univ. of Massachusetts Dairy Club held its Third Annual Dairy Club Alumni Breakfast. Over 60 persons attended the breakfast and took part in Homecoming Day activities in the afternoon.

Two events spotlighted the Breakfast program. The first was the recognition of Joseph V. Ziomek, an outstanding alumnus, for his service to the dairy industry in Massachusetts. Mr. Ziomek, now superintendent of production for Bushway Ice Cream Co. in Somerville, Mass., graduated from the Stockbridge School of Agriculture in 1930. Since graduation, he has been employed continuously by the same firm. The following is quoted from his citation: "During these 26 years, he has trained many young men and has taken part in at least two important recent advances made by the industry, namely Automation and Cleaning in Place. He has developed numerous devices as production aids which attest to his ingenuity and production skill."

The second event was the formal presentation by the General Ice Cream Corp. to the Univ. of Massachusetts of a modern 3-ft. stainless steel vacuum pan. Earl R. Stevens, representing the General Ice Cream Corp., presented Dr. Shannon McCune, Provost of the University, with an engraved plaque for mounting on the equipment.

Masters of ceremony for the Breakfast were Robert N. Bairstow and James M. Sullivan, co-presidents of the Massachusetts Student Chapter of A.D.S.A.

Minnesota Dairy Science Club Activities

The Univ. of Minnesota Dairy Science Club held its annual student-faculty smoker on Oct. 25, with 45 persons attending. The purpose of this event is to provide an opportunity for faculty and students to become better acquainted, and to acquaint new students with the facilities available in the Dairy Department. C. L. Cole, the new department head, was introduced to the club, along with the faculty, graduate students, scholarship winners, and Dairy Cattle and Dairy Products Judging Team members. A tour of the Dairy Department facilities was conducted, and a lunch consisting of dairy products was served.

At its Nov. 13 meeting, Floyd Thompson, executive secretary of the Minnesota Creamery Operators & Managers Assoc., discussed "The Operation of Minnesota Creameries" and pointed out the ever present demand for well trained men in the dairy industry.

Ohio State Chapter News

Fifty persons attended the annual Student-Employer Appreciation Day program on Oct. 6. The program included a tour of the Dept. of Dairy Technology with research displays presented by the faculty.

Webb Jennings of Dairymens Ohio Farmers Milk Co., Cleveland, was the guest speaker. His topic was "Employer's Attitude Toward the Summer Employment Program." W. L. Slatter of the staff spoke on the current activities in the department.

A luncheon was served by the students, and the program was concluded by attending the Ohio State–Stanford football game.

The Dairy Club honored 35 freshmen at the annual Freshman Bean Feed on Oct. 16. The traditional football game was won by the freshmen over the upperclassmen, 22-0. After the game, supper was served to 65 students and fac-

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ulty. The functions of the Dairy Clubs were explained to the new students by officers of the clubs. At the completion of the program, new members were recruited for the A.D.S.A. Student Affiliate Branch.

Oregon Club Has Full Schedule

The Oregon College Dairy Club has conducted a full program of activities and projects this college term and has extensive plans for the remainder of the year. The Club helped finance the trip of the OSC dairy cattle judging team to Waterloo, Iowa, for the National Intercollegiate Dairy Cattle Judging Contest this fall. Team members were DAN BULLIS, SOLON SPENCER, DELBERT MAYER, and alternate AUSTIN CONNOLLY. JOHN ALHSTROM, AUSTIN CONNOLLY, and FRANK PELLEGRINO were members of the dairy cattle judging team who participated in the regional dairy show at the Cow Palace in San Francisco in early November. The club financed the transportation and lodging for the trip. Oregon State College entered both a dairy products and dairy cattle judging team at the Pacific International Livestock Exposition in Portland, Oregon, on Oct. 20.

A Committee is working on the selection and purchase of a purebred dairy heifer at a future sale or dairy show. This investment is being made as a fund-raising project, through the sales of future offspring of the club animal.

Several leaders of the dairy industry are scheduled to speak at future club meetings. At an early date a representative of the Carnation Company will speak on the topic, "What the dairy industry expects of a college graduate."

Tennessee Has Top Dairy Cattle Judge at Waterloo

Harold Childress of the U.T. Dairy Cattle Judging Team won top honors in judging Guernseys and was first in judging all five leading dairy breeds at the annual judging contest held in conjunction with the International Dairy Congress at Waterloo, Iowa. The team was coached by S. A. Hinton, professor of dairy husbandry. Harold, having been reared on a dairy farm, got off to an early start in the cattle business. At the age of twelve, he owned his first calf and he has grown a number of animals since then. He has been a 4-H Club member for 8 years.

Glenn Parks of Fayetteville, Tenn., participated in the summer Guernsey training program and worked at the Quail Roost Guernsey Farm, Rougemont, N. C., this past summer.

The Dairy Club entertained 28 dairy alumni at the Annual Milk Hour preceding the Homecoming game Nov. 3. During the day, the club also assisted with a program for 1,100 agriculture and home economics high school students and their advisors.

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THE MECHANICS OF A CREAM SEPARATOR CLEANED AND STERILIZED BY CENTRIFUGAL FORCE

E. O. HERREID AND E. SVEREIKA[1]

Department of Food Technology, University of Illinois, Urbana

A self cleaning separator is in keeping with the modern trend toward automation. The authors attempt to explain the mechanics of a cream separator cleaned and sterilized by centrifugal force. *Editor.*

When milk revolves in a separator bowl the cream is accelerated toward the center by a force equal to $m \frac{V^2}{R}$, where V is the velocity and R is the radius. This is the centripetal force and is such that $F = m \frac{V^2}{R}$. Against this force the skimmilk exerts an equal and opposite centrifugal force, but because of its higher density it is thrown tangentially outward and farthest from the axis of the bowl. This principle was applied in attempts to develop a cream separator in Germany nearly 100 years ago *(1, 2)*, and it will be used to explain the mechanics of a cream separator cleaned and sterilized by centrifugal force.

Description of separator. The bowl consists of the base, the shell, the skimming discs, the top disc, the flexible bowl ring, and the steel snap ring (Figure 1). The bowl base has three posts located at 120° intervals in the outer circumference. Each disc is held in place by three notches which fit tightly against each post. The flexible bowl ring is tightly stretched over the outside of the posts (Figure 2) and it is made of steel, wound spirally and covered with rubber. The top disc fits against the separating discs. The bowl shell is held to the bowl base by a steel snap ring that slips into a groove underneath the base. The snap ring expands into the groove when the bowl spins. The bowl shell has a row of horizontal openings around its shoulder and the flexible ring closes them as the bowl attains normal velocity. The base of the cream assembly fits tightly into the frame of the machine and has a rubber gasket in a groove which seals against the skimmilk assembly, which in turn has a rubber gasket set in a groove against the cover. The cover has a rubber gasket which seals against the discharge valve from the supply tank (Figure 3). The supply tank is mounted on an adjustable support which can be raised or lowered by a pedal. A toggle clamp is anchored on the frame and its free end is clamped to a bolt head on the post that supports the tank. When the toggle clamp is closed, the cream and skimmilk assembly is tightly sealed to the outlet from the tank, thus preventing

Received for publication April 16, 1956.

[1] International Harvester Company, McCormick Works, Chicago, Ill.

FIG. 1. Showing different parts of the bowl.

leakage of solutions. The supply tank has a valve to meter out the milk and one to release cleaning and sterilizing solutions (Figure 4).

EXPERIMENTAL RESULTS

The rubber covered steel ring in the bowl is the device that makes it possible to clean this separator by centrifugal force. This ring can expand and contract, depending on the velocity of the bowl. At the normal bowl velocity, the ring fits tightly against the horizontal openings at the shoulder of the bowl shell, but

FIG. 2. Showing base, discs, and flexible bowl ring in place.

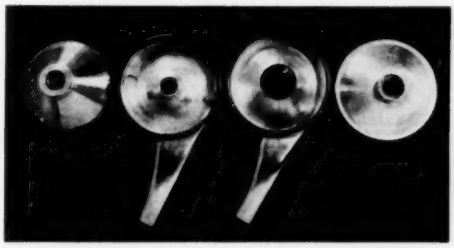

FIG. 3. Showing bowl, skimmilk and cream collectors, and cover with gaskets.

when the velocity decreases the ring relaxes and cleaning solutions can flow through all parts of the separating mechanism at considerable pressures. Since the revolving bowl creates the pressure that forces cleaning and sterilizing solutions through the cream and skimmilk assemblies, it was decided to determine the pressure in the bowl and its velocity the instant that solutions are released.

Determining the pressure. The first step was to determine the bowl speed at which the ring seals against the openings in the shell. A steel spring of the type used in the bowl ring was tested on a tensile test machine and a load versus elongation in in/in. was plotted. It was next necessary to determine the tension in the spring after it was mounted on the expander wire. This tension was found to be 21.5 lb. from the graph for the amount of deflection required to assemble the spring over the expander wire in the proper position. From a drawing the increase in radius required to seal the bowl ring was found to be 0.178 in. This deflection was added to the elongation already existing in the spring at zero r.p.m., and a tensile load of 71.5 lb. was found in the spring according to the graph. The calculations are as follows:

Spring circumference before mounting on expander wire is:

$$15.875 - 4(0.055) = 15.655 \text{ in.} \qquad (1)$$

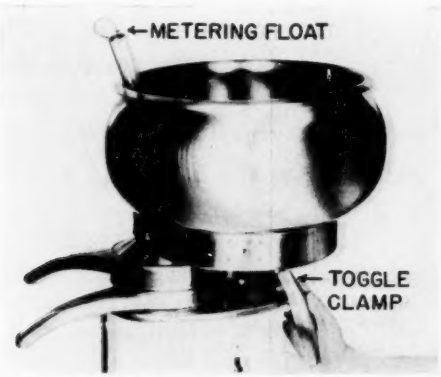

FIG. 4. Separator showing toggle clamp that holds the outlet from supply tank tightly against the cream and skimmilk assembly.

Increase in circumference when spring is mounted on expander wire is:

$$5.060\,\pi - 15.655 = 0.245 \text{ in.} \tag{2}$$

From a drawing, it was found that the radius increases 0.178 in. when ring seals. Therefore, spring length is increased by:

$$2\,\pi\,(0.178) = 1.12 \text{ in.} \tag{3}$$

Radius when ring is sealed = 2.708 in.

$$\text{Deflection of spring at zero r.p.m.} = \frac{0.245}{15.655} = 0.01565 \text{ in/in.} \tag{4}$$

Spring tension at zero r.p.m. = 21.5 lb. (Taken from load-deflection curve)

$$\text{Additional deflection when ring seals} = \frac{1.12}{15.655} = 0.0716 \text{ in/in.} \tag{5}$$

Then 0.07160 + 0.01565 = 0.08725 in/in., total deflection when ring seals.

Spring tension from graph when ring seals = 71.5 lb.

Next, a number of bowl rings were weighed, and the mean weight per unit length of spring was determined. By using the centrifugal force equation, $F = \frac{WV^2}{GR}$, the force acting on a unit length of spring was calculated. Then applying the so-called "hoop stress" equation, an expression for the tension in the spring due to centrifugal force was derived. This expression was equated to the tension known to exist in the spring and solved for r.p.m. The calculations are as follows:

48.3 g. = mean weight of rings. Weight of bowl ring/in. of spring length,

$$\frac{48.3}{2\,\pi \times 2.708} = 2.84 \text{ g. or } 0.00623 \text{ lb.,} \tag{6}$$

Let F = centrifugal force acting on 1 in. of bowl ring.

$$\text{Then, } F = \frac{WV^2}{GR} = \frac{W\,(2\,\pi NR)^2}{GR} = RW\,\frac{(4\,\pi^2)}{G}\,N^2 \tag{7}$$

$$F = \frac{0.00623 \times 4 \times (3.14)^2 \times 2.708\,N^2}{386} = 0.001728\,N^2 \text{/in. of spring} \tag{8}$$

Then, tension in spring due to centrifugal force =

$$FR = 0.001728\,N^2 \times 2.708 = 71.5 \text{ lb.} \tag{9}$$

$N^2 = 15{,}326$, $N = 123.8$ r.p.s., $N = 7{,}428$ r.p.m.

The speed at which the bowl ring should seal was found to be 7,428 r.p.m., which is theoretical, because it was assumed that the ring would seal as soon as it touched the horizontal slots in the bowl shell. Actually, the bowl ring is probably slightly compressed against the slots before a tight seal is obtained.

It was desired to determine the revolutions per minute and pressure at the instant the bowl ring relaxes and opens the slots. This was done by equating

the total spring tension to the sum of the tensions due to centrifugal force and the tension due to the fluid pressure acting on the bowl ring.

The fluid pressure equation was derived for the pressure of a rotating fluid as follows:

$$Y = \frac{W^2 r^2}{2g} + C, \text{ where } Y = \text{height of rotating fluid column at radius } r. \tag{10}$$

$$\text{When } Y = O,\ r = r_1, \text{ and } C = -\frac{W^2 r^2}{2g} \tag{11}$$

$$\text{Therefore, } Y = \frac{W^2}{2g}(r^2 - r_1^2) \text{ (Figure 5)} \tag{12}$$

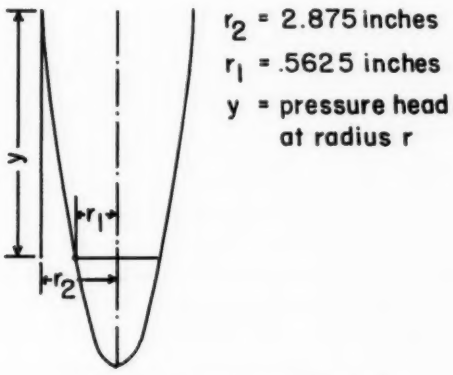

FIG. 5. Rotating fluid in center of bowl.

Then, substituting P for Y and introducing ρ into the equation, the pressure can be calculated as follows:

$$P = \frac{\rho W^2}{2g}(r_2^2 - r_1^2) \text{ in which} \tag{13}$$

ρ = fluid density = lb/in.^{3} = 0.0385 lb.
P = pressure at radius r_2.
r_1 = inner radius of fluid column as determined from drawing of bowl (Figure 5).
r_2 = outer radius from drawing.
g = gravitational constant = 386 in/sec^2.
W = angular velocity in rad/sec.

$$\text{Then, } P = 0.0385\ W^2 \frac{[(2.875)^2 - (0.5625)^2]}{2 \times 386} = 0.000397\ W^2 \tag{14}$$

Force on bowl ring per unit length due to fluid pressure =

$$\frac{P \times \text{area of opening sealed by bowl ring}}{\text{circumference of spring}} = F$$

$$F = \frac{0.000397\ W^2 \times 5.75 \times \pi}{8 \times 2.708 \times 2\pi} = \frac{0.000397\ W^2 \times 5.75}{16 \times 2.708} = 0.0000528\ W^2 \quad .. \quad (15)$$

Additional spring tension due to fluid pressure. (Hoop stress equation)

$$\text{Tension} = 0.0000528\ W^2 \times 2.708 = 0.0001428\ W^2 \quad (16)$$

Total spring tension = tension due to fluid pressure + tension due to centrifugal force.

$$\text{Total spring tension} = 71.5 = 0.0001428\ W^2 + 0.001728 \times 2.708\ N^2 \quad (17)$$

Expanding eq. 16 $= 0.0001428\ (39.5)\ N^2 + 0.00466\ N^2$

and eq. 9 $= 0.00564\ N^2 + 0.00466\ N^2$

$W = 2\pi N$ $\qquad N^2 = 6{,}955$

$W^2 = (2\pi N)^2$ $\qquad N = 83.4$ r.p.s.

$= 39.5\ N^2$ $\qquad N = 5{,}004$ r.p.m.

$P = 0.000397\ W^2$

$= 0.000397\ (83.4 \times 6.28)^2 \quad (18)$

$= 109$ p.s.i.g. when the ring relaxes.

Thus, the pressure is 109 p.s.i.g. at the instant that the ring relaxes in the bowl. The pressure is zero when the ring seals, because there is no fluid in the bowl. The value, 5,004 r.p.m., is very close to the actual test results.

SUMMARY

It was found at a velocity of 5,004 r.p.m., that the bowl released cleaning and sterilizing solutions into the cream and skimmilk assembly at 109 p.s.i.g., which is the pressure the instant the solutions leave the bowl.

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THE SANITARY ASPECTS OF A CREAM SEPARATOR CLEANED AND STERILIZED BY CENTRIFUGAL FORCE

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The results of a number of trials indicate that a centrifugal separator can be cleaned and sterilized efficiently by centrifugal action without disassembling the machine. Similar results can be expected from a factory separator. *Editor.*

Cleaning equipment and pipe lines without disassembling them is an accepted practice in the dairy industry in this country. The commercial clarifier and cream separator are disassembled for cleaning.

A study was made of the sanitary efficiency of cleaning and sterilizing a cream separator by centrifugal force. The pressure in the revolving bowl was found to be 109 p.s.i.g. at the instant the solutions were released into the cream and skimmilk assembly. The description of the separator and its mechanics has been reported *(2)*. A similar study was made of another separator *(3)*.

PROCEDURES

Cleaning. The detergents were placed in the supply tank with 2½ gal. of water at 120-125° F. When the bowl attained its maximum velocity, about one-third of the solution passed through the cream and skimmilk assembly. Then the power was shut off, and the bowl velocity decreased until the flexible rubber covered steel ring relaxed. At this instant another third of the solution passed through and the power was turned on. The bowl velocity increased, but not enough for the flexible ring to close the openings in the bowl shell, and the last third of the solution passed through the machine followed by a quart of water. Finally, the cream and skimmilk assembly was dried by allowing the bowl to attain maximum velocity and then shutting off the power.

The detergents used are designated by number, and their composition follows:

1. Na_2CO_3, 47.6%; Na_3PO_4, 9.5%; Na_2SiO_3, 7.6%; $Na_4P_2O_7$, 25.7%; wetting agent, 4.8%; and H_2O, 4.8%.
2. Na_3PO_4. 10 H_2O.
3. Sodium salt of a sulfated monoglyceride, 43%; $Na_4P_2O_7$, 9%; Na_2SO_4, 45%; H_2O, 3%. Figures are averages of a range given by manufacturer.
4. pH neutral, sodium alkyl benzene sulfonate, Na_2SO_4, NaCl, H_2O, minor ingredients.
5. Organic acid — composition not available.

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The detergents were used in concentrations of 0.1 to 0.2%, as recommended by the manufacturer.

Sterilization. This means practical sterilization and not the complete destruction of all microorganisms. Two procedures were used to sterilize the separator before it was used. In method 1, 2½ gal. of water was passed through the machine and discharged at 170° to 175° F., and in method 2, the same volume of water at 115° to 120° F. containing 200 p.p.m. of chlorine was used. The solutions were passed through the machine in the same way as the detergent solutions. In some experiments the machine was sterilized only at the beginning of the experimental trial and not before every separation. The containers for the cream and skimmilk were allowed to remain in contact with water at 170° to 175° F. for about 5 minutes. The hardness of the water varied from 183 to 196 p.p.m.

Bacteriological analysis. The samples for bacteriological analysis were taken with sterile pipettes and placed in sterile tubes in an iced bath and were plated in duplicate within 2 hours according to standard methods *(1)*. The samples for plating were warmed to 95° to 100° F. in 3 to 5 minutes, dilutions were made immediately, and the medium was poured into the plates within 10 to 15 minutes after the first warming.

Separations. Twenty gallons of milk containing 3.8 to 4.2% fat, obtained from the University Farm, was used. The milk was well mixed, and half of it was separated in the morning and the other half was held at 40° to 45° F. and separated in the late afternoon. The separations were made at 95° to 100° F. on consecutive days for either 3½, 7, or 14 days, and one trial lasted for 30 days. One quart of skimmilk from each separation was used to remove sediment in the bowl after the flexible ring had relaxed and was collected in the cream and in the skimmilk.

Determining the sanitary efficiency of the separator. The sanitary efficiency of the separator was determined by multiplying the plate counts by the entire volume of cream and of skimmilk, and comparing them with the total plate count for all of the milk. For example, the plate count of the milk was 5,600 per cc. (Trial 1, Table 1), then for all of the milk it was $37{,}836 \times 5{,}600 = 211.8$ millions; for the skimmilk, $33{,}078 \times 6{,}800 = 244.9$ millions, and for the cream $4{,}740 \times 3{,}200 = 15.1$ millions (Trial 1, Table 2). The volumes used for these calculations were obtained by separating seven lots of milk of 85.5 ± 0.1 lb. each, obtaining an average of 10.46 ± 0.03 lb. of cream and 75.04 lb. of skimmilk calculated by difference. The avoirdupois weights of the three products were converted to metric weights and then to metric volumes, by using specific gravities of 1.025, 1.001, and 1.028 for milk, cream, and skimmilk, respectively. The Westphal balance was used to estimate specific gravity at $\frac{97.5}{77.0} \pm 0.2$ F.

Determining surface areas. A centrifugal cream separator has much internal surface which contacts milk, cream, or skimmilk. The surfaces of all parts were measured and calculated to obtain the total surface. The skimming dises, the

TABLE 1

Mean plate counts of milk, and of cream and skimmilk obtained from it, when the separator was cleaned and sterilized

Trial No.	No. of separations	Detergent used	Sterilizer used	Milk	Cream	Skimmilk
				Plate counts/cc.		
1	7	1	Cl	5,600	3,200	6,800
2	7	1	H_2O	6,700	3,900	8,700
3	7	2	Cl	5,200	3,500	7,300
4	7	2	H_2O	5,000	2,800	5,000
5	7	3	Cl	5,100	2,900	6,200
6	7	3	H_2O	6,000	3,500	6,200
7	7	4	Cl	11,900	5,900	12,000
8	7	4	H_2O	10,300	6,300	13,600
9	7	3	H_2O[a]	7,000	3,100	7,300
10	60	3	Cl	7,600	3,800	7,500

[a] Distilled water was used for both the detergent and sterilizing solutions.

top disc, and parts of the bowl shell, bowl base, and circular part of the cream, skimmilk, and cover assembly were assumed, for the most part, to have the shape of a frustum of a right circular cone with larger lower and smaller upper radii. The total surface of each of these parts was calculated according to the equation:

$$\text{Lateral area} = \pi S(r + R)$$
$$= \pi(r + R)\sqrt{(R - r)^2 + h^2}$$
$$S = \sqrt{(R - r)^2 + h^2} \text{ in which}$$

S = slant height

h = height

R and r = radii of bottom and top, respectively.

The areas of the three holes and of the three notches in the skimming discs were deducted. The lateral areas at the top and the bottom of the discs were measured and calculated. The top disc has three ridges located at 120° around the circumference and they were assumed to be isosceles trapezoids.

The circular part of the cream spout is a frustum with the top and bottom surfaces having the same area. The horizontal ring at the bottom has an inner and an outer surface, which have the same area. The vertical ring, which fits

TABLE 2

Mean distribution of the total plate counts of all the milk, and of all the cream and skimmilk obtained from it, when the separator was cleaned and sterilized

Trial No.	No. of separations	Milk 37,836 cc.	Cream 4,740 cc.	Skimmilk 33,078 cc.	Cream	Skimmilk
		Total plate count 1×10^6			% of plate count of milk	
1	7	211.8	15.1	244.9	7.1	106.2
2	7	253.5	18.5	287.8	7.3	113.5
3	7	196.7	16.6	241.5	8.4	122.8
4	7	189.2	13.3	165.4	7.0	87.4
5	7	192.9	13.7	205.1	7.1	106.3
6	7	227.0	16.6	205.1	7.3	90.4
7	7	450.2	28.0	396.9	6.2	88.2
8	7	389.7	29.9	449.9	7.7	115.4
9	7	264.8	14.7	241.5	5.6	91.2
10	60	287.5	18.0	248.1	6.3	86.3

tightly into the frame of the separator, has one exposed surface. The groove for the rubber gasket has a lateral and a vertical surface. The surfaces of the skimmilk spout were calculated in essentially the same way.

The spout assembly cover is the shape of a frustum with an outside groove which holds the rubber gasket and a center groove and a rubber gasket which seals the cover against the supply tank.

The bowl base has a top and a bottom frustum, an inside and an outside rim, and three posts at 120° intervals in the circumference to hold the discs. The bowl shell also has the shape of a frustum with top and bottom surfaces with inner and outer horizontal and vertical bands of metal. The areas of the horizontal discharge ports on the shoulder of the shell were calculated and deducted from the total area.

The metering valve assembly in the supply tank has a float, including a ring, a stem, a rubber roller metering device, a ground valve with a horizontal rod support, a side wing, and four prongs. It was necessary to use a planimeter to calculate the surfaces of some of these parts.

Since the supply tank and its lower gasket surface were composed of essentially circular parts of definite heights, the surfaces were not difficult to measure and calculate. The calculations are summarized in Table 5.

RESULTS

The results of 10 trials and a total of 123 separations are summarized in Table 1. In all trials the separator was sterilized immediately before it was used. Detergent 1 and chlorine sterilization were used in Trial 1. After seven separations the upper part of the skimmilk assembly had a film of solid material on it. The inside surface of the bowl shell and its base had an adsorbed layer of material. All discs had thin films of white material on their underneath surfaces. The acid detergent removed most of this material. In Trial 2, detergent 1 and water sterilization were used. All separating parts were clean except that the underneath surfaces of the discs had very slight films, which were removed with the acid detergent. In Trials 3 and 4, detergent 2 and chlorine and water sterilization were used. All parts were free of solids except that the underneath surfaces of the discs had thin loosely held films, which were easily rubbed off and were removed by flushing the acid detergent through the separating parts. In Trial 5 detergent 3 and chlorine sterilization, and in Trial 6 the same detergent and water sterilization were used. Again, all parts were free of milk solids except that the underneath surfaces of the discs had slight films, which were with the acid detergent. In Trials 3 and 4, detergent 2 and chlorine and water sterilization and detergent 4 were used. The results were the same as for trials 6 and 7. In Trial 9 distilled water was used for both the detergent and sterilizing solutions. The discs and all surfaces of the bowl were clean and free of films of solids.

Trial 10 represents 60 separations for 30 consecutive days, in which detergent 2 and chlorine sterilization were used. Examination of the separator after 7 days

showed that all parts were clean except the underneath surfaces of the discs, which had very slight films. An examination of all parts after operating the machine for 14 days showed about the same conditions as were observed after 7 days, except for some lightly adsorbed solids around the lower edge of the bowl shell and in the upper part of the skimmilk. All parts were examined again after 21 days, and a small spot of solid material was found on the center of the cream assembly. The discs had slight films of adsorbed materials on their underneath surfaces. After 30 days this trial was concluded and all parts were examined. There was a slight layer of lightly adsorbed material on the underneath surfaces of the discs. Small specks of solids adhered to the center ring of the cream assembly. The milk and cream spouts had small areas of lightly adsorbed solids in their inner surfaces. The bowl shell was clean, but the bowl base had some solid material where the steel ring snaps into the groove; however, this is not in the path of cream or skimmilk. The rubber gaskets were clean and odorless. Flushing 2½ gal. of an acid detergent through the machine removed adsorbed solids on all surfaces.

In spite of the inherent variability of the plate count method, the results in Table 2 indicate fair agreement in comparing total plate counts in the cream and skimmilk with those in the milk. It can be assumed that contamination from the machine was negligible. Furthermore, the plate counts for all of the cream varied within only 5.6 to 8.4% of those for all of the milk. The plate counts per cubic centimeter of the cream were slightly more than one half those for the milk (Table 1). This criterion for evaluating the cleaning efficiency of the separator was compared in trials where the machine was not sterilized before each separa-

TABLE 3

Mean plate counts of the milk, and of cream and skimmilk obtained from it, when the separator was cleaned but not sterilized

Trial No.	No. of separations	Milk	Cream	Skimmilk
		Plate count/cc.		
11	14	13,500	15,900	18,900
12	14	8,500	10,100	9,700
13	14	24,300	31,800	30,200
14	14	59,000	63,000	56,000
15	14	78,000	91,600	105,000
16	14	113,000	205,000	180,000

TABLE 4

Mean distribution of the total plate counts of all the milk, and of all the cream and skimmilk obtained from it, when the separator was cleaned but not sterilized

Trial No.	No. of separations	Milk 37,836 cc.	Cream 4,740 cc.	Skimmilk 33,078 cc.	Cream	Skimmilk
		Total plate counts 1×10^6			% of plate count of milk	
11	14	510.8	753.7	625.2	147.5	122.4
12	14	321.6	478.7	320.8	148.8	99.8
13	14	919.4	1,507.3	998.9	163.9	108.6
14	14	2,232.2	2,986.2	1,852.4	133.8	83.0
15	14	2,951.2	4.341.8	3,473.2	147.1	117.7
16	14	4,275.5	9,717.0	5,954.0	227.3	139.3

TABLE 5

The area of all parts of the centrifugal separator that contact milk, cream, or skimmilk

Name of part	Surface area ($cm.^2$)
Bowl base	401.9
Bowl shell	557.0
Skimming discs, 22	3,201.0
Upper disc	359.5
Cream collecting assembly	1,353.8
Skim collecting assembly	1,200.4
Cover, skim assembly	636.3
Metering assembly	380.5
Milk tank	1,915.7
	10,006.1 $cm.^2$ = 1,550.9 $in.^2$ = 10.8 $ft.^2$

tion. In these trials (Table 3) the plate counts of the cream and skimmilk exceeded those of the milk in practically all cases and the calculated distribution of the total plate counts in both products (Table 4) indicated considerable contamination from the machine.

DISCUSSION

The results of this study indicate that the separator can be cleaned and sterilized efficiently by centrifugal force and that similar results can be expected from a factory separator, provided such equipment becomes available. The adsorption of thin films, presumably residues from detergents and from salts in the water, did not affect the bacteriological quality of the cream and skimmilk. After rinsing and washing, except in Trial 9 (Table 1), there was always a slight residue which had to be removed with the acid detergent. There was no residue after completing Trial 9, suggesting that salts in the water may have been partly responsible for this condition. It is suggested that an acid detergent be used daily after washing with the alkaline detergent.

The stepwise procedure for cleaning and sterilizing the separator should be followed in detail and be done immediately after completing every separation because the revolving bowl tends to remove moisture and thereby leave, on the metal surfaces, milk solids which make cleaning more difficult if delayed.

SUMMARY AND CONCLUSIONS

The cream separator can be cleaned and sterilized by the centrifugal force of a bowl revolving at a velocity of 5,024 r.p.m., releasing solutions into the cream and skimmilk assembly at a calculated pressure of 109 p.s.i.g.

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A STUDY OF THERMAL RESISTANCE OF MICROCOCCI IN MILK[1, 2]

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The control of micrococci in pasteurized milk and milk products is important in the marketing of high quality dairy products. Fifty-one cultures, isolated from raw milk and from laboratory pasteurized milk supples, were studied to determine their thermal death times at 143° F., and thermal death time curves were established for selected cultures. Uniformity in heat resistance from one determination to the next is the exception rather than the rule. The findings in this study do not support the common conception that the cow's udder is an important habitat of thermoduric micrococci. *Editor.*

Relatively few studies have been made of the time of exposure required at different temperatures to destroy bacteria that are commonly found in milk and milk products. The wide range of temperatures used in processing dairy products suggests a need for more information in this regard.

Interest in thermoduric bacteria has been due, primarily, to (*a*) the introduction of more stringent bacterial standards on pasteurized products; (*b*) the adoption of a more favorable medium and temperature of incubation for the standard plate count which has resulted in obtaining higher bacterial counts; and (*c*) the increased use of the high-temperature, short-time system of pasteurization which has been shown *(1, 15, 18, 20, 21, 30)* to be generally less efficient in bacterial destruction than the low-temperature, holding method. Among the thermoduric bacteria that are commonly found in raw milk, certain species of the genus *Micrococcus* are almost invariably present *(4, 11, 13, 16, 17, 22, 25, 27)*. Consequently, they may contribute to difficulties experienced by milk processors in maintaining bacterial counts of pasteurized milk products within legal limits. It has been recently emphasized *(2)* that thermoduric bacteria do not increase significantly in pasteurized milk during storage at temperatures of 7.3° C., or below, but do increase rapidly during storage at 10° C. These organisms, therefore, may be responsible for the deterioration of pasteurized products, for these products frequently may be held at temperatures above 7.3° C. Further, since micrococci are natural inhabitants of the cow's udder *(6, 10, 12, 14)* and since they are important contaminants on dairy utensils *(23, 24, 29)*, they are of concern to producers in controlling the bacteriological quality of the raw milk.

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[2] Taken from data presented in a thesis submitted to the graduate faculty of the University of Minnesota by A. N. Myhr in partial fulfillment of the requirements for the Master of Science degree.

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The purpose of this investigation was to provide additional information with regard to:

1. The thermal death time characteristics of micrococci in milk.
2. The thermal tolerance of micrococci in milk drawn aseptically from the udder.
3. The variation in heat resistanee of different cultures of micrococci.

EXPERIMENTAL METHODS

In the selection and isolation of cultures, 10 ml. of sample were collected from various patrons' raw milk at the weigh tank and transferred to sterile test tubes (150 × 16 mm.). The portion of the tube extending above the level of milk was flame sterilized. The samples were laboratory pasteurized at 143° F. for 30 minutes, after which they were cooled and plated in tryptone glucose beef extract milk (TGE) agar. The plates were incubated at 35° C. for 48 hours. Portions of well isolated colonies were transferred to tryptone glucose beef extract (TGE) broth and incubated at 35° C. for 24 hours. The cultures which appeared as coccus forms with a tendency to form clumps were retained for purification and study.

In order to investigate the cow's udder as a potential source of thermoduric micrococci, milk samples were collected aseptically by thoroughly cleaning the udder with warm water, after which the teats were disinfected with an aqueous solution containing 200 p.p.m. of a quaternary ammonium compound. After 10 to 12 streams of the foremilk had been discarded, samples were drawn directly into sterile screw-capped test tubes. Usually, the milk from all four quarters was composited. In most instances the samples were heated for 30 minutes at 143° F. before isolations of thermoduric micrococci were attempted.

The cultures of micrococci used in heat resistance trials were carried in litmus milk and transferred every 2 or 3 weeks. After each transfer, the cultures were incubated for 24 hours at 35° C. and stored at approximately 5° C. between transfers.

Preparatory to heat treatment, each culture was grown through two successive transfers in litmus milk at 35° C. for 24 hours per transfer. One hundred ml. of sterile skimmilk (containing glass beads) was inoculated with a loopful of the second transfer. After 24 hours incubation, the culture was shaken vigorously 75 to 100 times to assist in breaking up bacterial clumps. After the shaking, an amount of inoculum previously determined to yield approximately 100,000 to 200,000 bacteria per milliliter was transferred to 100-ml. amounts of cold sterile methylene blue milk. The suspension was shaken 25 times and dispensed in 2-ml. amounts into replicate 10 × 75 mm. test tubes. These tubes were heat sealed and completely immersed in a constant temperature water bath for heat treatment. After the desired period of exposure at a given temperature, a series of tubes were withdrawn from the bath and cooled immediately in flowing tap water at approximately 15° C. The tubes then were incubated at 35° C. for periods up to 20 days. Reduction of the methylene blue milk was used as a

measure of viability; however, when weakly reducing cultures were encountered, survival was ascertained by aseptically breaking the tubes and streaking a portion of the contents on TGE agar.

The experimental heating times of the contents in the thermal death time tubes were corrected in accordance with the procedure of Olson *et al. (19)* and Slatter and Halvorson *(26)*, to compensate for heating lag.

RESULTS

Fifty-one cultures, some isolated from raw milk drawn aseptically from cows, and some from laboratory pasteurized raw milk supplies, were studied to determine their thermal death times at 143° F. The results of this survey are recorded in Table 1. All 12 cultures isolated from raw milk aseptically drawn from the

TABLE 1

Thermal death times of micrococci at 143° F.

Source	< 5	5-10[a]	10-15	15-20	20-25	25-30	> 30	Total time
				(*min.*)				
Raw milk aseptically drawn	12							12
Lab. past. milk	9	6	5	3	1	2	13	39

[a] 5-10 = survived 5 but not 10 minutes, etc.

udders of cows were destroyed in less than 5 minutes at this temperature. Of the 39 cultures isolated from laboratory pasteurized samples of milk only 13, or one-third, survived after they had been subcultured and repasteurized at 143° F.

In further studies of the cow's udder as a possible source of thermoduric micrococci, 51 samples of milk were collected aseptically from one or more quarters of 26 cows in the University of Minnesota herd and from 12 cows in a local herd. The samples were subjected to 143° F. for 30 minutes and plated. Very few colonies developed on the plates prepared from the samples. Microscopic examination of the organisms which survived showed these to consist largely of rod types, and occasionally a member of the genus *Streptococcus* was found. In only one instance was a thermoduric species of the genus *Micrococcus* recovered. Since this organism was represented by only one colony on the agar medium, it might have been an external contaminant.

Raw milk frequently is held on the farm or in the plant for as long as 24 to 36 hours before it is processed. It was thought that this may, in some way, affect the susceptibility of the bacterial flora to destruction by heat. Milk samples from seven cows, therefore, were divided; one portion of each was heated at 143° F. for 30 minutes shortly after they were obtained from the cows and the second portion after 30 hours storage at 5° C. The plates prepared from these samples were negative for micrococci, indicating that if storage had increased the heat resistance of the organisms, it had not done so to an extent which would enable the organisms to survive pasteurization.

For the purpose of further characterizing thermal death times of cultures of micrococci reported in Table 1, four isolates from laboratory pasteurized samples of raw milk supplies which survived 30 minutes at 143° F. were selected for study at temperatures other than 143° F. Two of these cultures, designated as P29 and II, were exposed to four different temperatures, and the remaining two, V and VII, were subjected to two temperatures only. The data are summarized in Table 2.

TABLE 2

Summary of heat resistance studies of four cultures isolated from pasteurized milk

Culture designation	Trial No.	Thermal death time in minutes at				Population per ml.	Approx. z value[b]
		148° F.	151° F.	155° F.	160° F.		
P29	1	80-85[a]	43-45	24-26	18-19	150,000	17.5
	2	> 90	> 50	> 32	> 20	130,000	——
	3	90-100	48-51	30-34	19-20	160,000	18.2
	4	> 100	51-60	> 34	20-22	62,000	——
	5	110-120	> 70	30-34	20-22	66,000	16.5
	6	110-120	> 70	> 40	22-25	150,000	——
	7	110-120	> 70	34-40	20-22	150,000	16.5
	8	120-130	> 85	45-50	20-22	170,000	16.0
II	1	84-90	52-56	27-30	10-11	130,000	13.0
	2	90-100	60-64	30-32	12-14	160,000	13.9
V	1	30-40	——	10-15	——	8,600	——
VII	1	15-20	——	5-10	——	290,000	——

[a] 80-85 = survived 80 but not 85 minutes, etc.
[b] z value = °F. required to effect a 10-fold change in thermal death time.

For illustrative purposes, only data from Trials 3 and 5 for culture P29 (Figures 1 and 2), and Trials 1 and 2 for Culture II (Figures 3 and 4) are plotted. From these curves (and from others not presented here) the z values or slopes were determined by noting the degrees Fahrenheit required to produce tenfold changes in thermal death times *(3)*.

It is evident from the data that there was considerable variation in thermal death times in some of the trials. The variations noted were most marked at the lower temperature ranges. The z values varied somewhat from one trial to

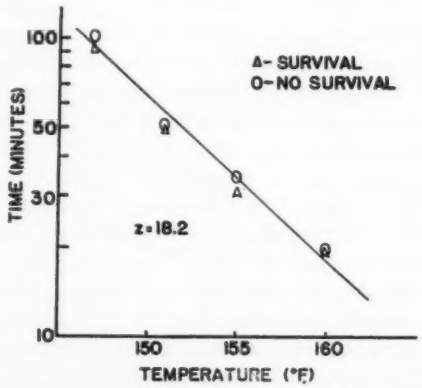

FIG. 1. Thermal death-time curve for Culture P29. (Trial 3, population 160,000 per ml.)

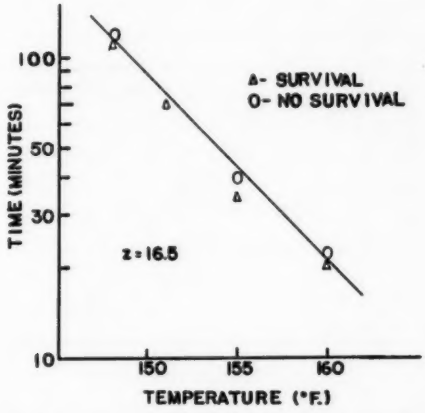

FIG. 2. Thermal death-time curve for Culture P29. (Trial 5, population 66,000 per ml.)

another but were observed to be consistently high. For instance, the z values obtained for culture P29 approximated twice that of the "pasteurization curve" (see Discussion), which has a value of 8.7. There was no correlation between the level of heat resistance and z values in the series of repeated experiments on culture P29. Data of Slatter and Halvorson *(26)* showed that z values were highest among the most heat-resistant species of lactobacilli used in their studies.

DISCUSSION

An interesting observation was made in connection with the cultures isolated from plates prepared from laboratory pasteurized milk. The majority of the cultures that survived the original laboratory pasteurization failed to withstand the same heat treatment after they had been isolated and subcultured. This may

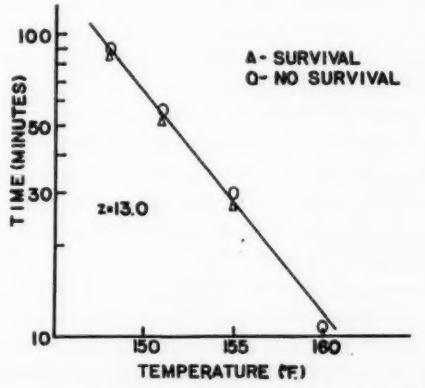

FIG. 3. Thermal death-time curve for Culture II. (Trial 1, population 130,000 per ml.)

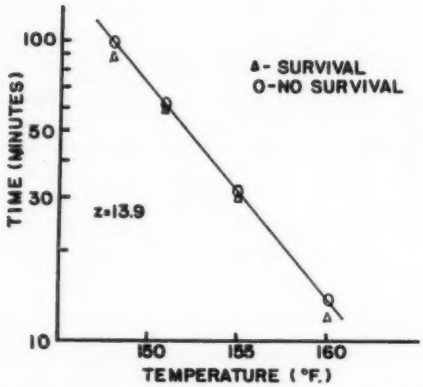

FIG. 4. Thermal death-time curve for Culture II. (Trial 2, population 160,000 per ml.)

have been due to physiological changes induced by passage through artificial media and sterilized milk in the purification and maintenance of stock cultures. Further, there is likelihood that the organisms were in a less active state in the cold milk as originally pasteurized than under the conditions of this study.

The heat resistance of micrococci varied somewhat in successive determinations throughout these studies. This also has been the experience of Speck and Lucas *(28)*, who reported a time range for the destruction of a species of *Micrococcus* at a given temperature rather than a specific destruction time. Carpentier and coworkers *(9)* were unable to obtain reproducible results in repeated trials on a group of freshly isolated micrococci. Barber *(5)*, however, did not encounter this variability in heat resistance with the species of *Micrococcus* used in his studies. It would appear, therefore, that uniformity in heat resistance from one determination to the next is the exception rather than the rule and that the results from a single determination should be interpreted with caution.

When variations in heat resistance were observed in repeated trials, the differences were greatest at the lower temperature ranges and diminished considerably as higher temperatures were reached. These disproportionate variations had the effect of producing changes in the slope or z values of thermal death time curves plotted from data obtained in individual determinations.

The reasons for variability in heat resistance within a species are not well known. There is the possibility of the occurrence of variant cells within a culture during subculturing. The tendency for certain bacteria, particularly the micrococci, to form masses or clumps of cells frequently has been reported to influence the end point of thermal destruction. Inherent errors in methods used to study heat resistance may account, in part, for apparent inconsistencies.

With widespread adoption of the high-temperature, short-time method of pasteurization, it frequently has been observed that the process was less efficient, from the standpoint of bacterial destruction, than the low-temperature, holding method. A plausible explanation for this phenomenon was advanced by Ball *(3)*

and further elucidated by Olson *et al. (19)*. This was based on the relation of thermal death time curves of bacteria to the pasteurization curve. The latter curve (obtained by joining two points representing 143° F. − 30 minutes and 161° F. − 15 seconds on semilogarithmic paper) has a z value of 8.7. If the thermal death time curve of a given bacterial culture shows a z value greater than 8.7 and if the curve is in a position near the pasteurization curve, the organism may be destroyed by the low-temperature, holding process but may survive the high-temperature, short-time method of pasteurization.

The data obtained in this investigation indicate not only the high heat resistance possessed by a number of members of the genus *Micrococcus* but that z values of their thermal death time curves are greater, in general, than have been reported for the non-sporeforming bacteria. For example, the z value of one species (P29) ranged from 16.0 to 18.2, which approximates values obtained for sporeformers.

Many authors have made reference to the cow's udder as an important habitat of thermoduric micrococci. The findings in this investigation do not support this conclusion.

The ever recurring reference in the literature to the cow's udder as a source of heat resistant micrococci stems largely from the work of Breed *(6)* who reported that 40% of the micrococci isolated from the udder were thermoduric. Other investigators *(10, 12, 14)* found that micrococci predominated in the udder flora but they did not study heat resistance. Still others have made studies of thermoduric micrococci from sources such as dairy utensils and pasteurized milk. These have been identified as to species, some of which have been given the same species designation as those isolated from udders by independent workers. Then, by simple analogy, the conclusion has been reached that the udder is a source of thermoduric micrococci. From the results of the present investigation, it does not appear likely that these organisms possess a high level of heat resistance during their existence in the udder. The possibility remains, however, that the bacteria may acquire increased thermal tolerance after exposure to the adversities of the outside environment. If utensils are not properly sanitized, the bacteria may grow in adhering surface films. A period of desiccation may ensue. Inefficient sanitizing procedures may exert a selective effect on the bacterial population, allowing subsequent enrichment of only the more resistant cells. Thus, through the processes of selection, enrichment, and possibly mutation, there may develop a strain of heat-resistant organisms from the less resistant parent strain that originated in the udder. It has been shown *(7, 8)*, however, that the udder may be invaded by thermoduric bacteria through improperly sanitized milking machines. The genera or species of these organisms were not specified. These invading organisms were not considered to be normal inhabitants of the udder, since, after the machines had been properly sanitized, the cows eliminated the organisms after 1 to 4 months. Under such conditions it also may be possible for thermoduric micrococci to invade and establish temporary residence in the udder.

SUMMARY AND CONCLUSIONS

Of 39 cultures isolated from laboratory pasteurized samples of raw milk from various patrons, only 13 survived 143° F. for 30 minutes after isolation and subculture in artificial media and sterilized milk.

The results indicate the high level of heat resistance possessed by certain members of the genus *Micrococcus*. The thermal death times of micrococci may be expected to vary considerably in successive determinations, particularly at the lower temperatures of exposure.

The z values or slopes of thermal death time curves for one species of *Micrococcus* studied were higher than those generally considered to be characteristic of nonsporeforming bacteria. In view of the explanation advanced by Ball *(2)*, the high z values obtained in this study might account for the lower bacteria-destroying efficiency of the high-temperature, short-time pasteurization process as compared to the low-temperature, holding method.

The data indicate that the cow's udder is not a common source of thermoduric micrococci. The possibility of micrococci acquiring increased heat resistance, after emission from the udder in the milk, was discussed.

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VARIATIONS IN ALKALINE PHOSPHATASE ACTIVITY OF MILK[1]

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Further information on variations in phosphatase activity in raw milk is given. The method used was a modification of that of Sanders and Sager. Wide variations in the phosphatase activity of milk from individual cows during one complete lactation period were noted. *Editor.*

All raw milk contains a phosphatase that can hydrolyze various phosphate esters under alkaline conditions. In 1933, Kay and Graham *(6)* described a procedure for determining phosphatase in milk and used it to study the stability of the enzyme at various temperatures. Since then several modifications have been developed for determining the amount of phosphatase in dairy products both before *(3, 4, 5, 7, 9)* and after *(1)* heat treatment.

Theoretically, variations in the initial enzyme concentration should yield corresponding variations in residual phosphatase in milks subjected to the same pasteurization treatment. However, comparatively little is known about the extent of variation of phosphatase activity in raw milk or the factors that influence it. Furthermore, it is difficult to compare available data because of differences in the experimental methods and the units used to express results. Folley and Kay *(3)* studied the relation between concentration of the enzyme and stage of lactation in Shorthorn and Guernsey cows in England. Kannan and Basu *(5)* attributed certain fluctuations in the phosphatase content of Indian cows to physiological disturbances. Limited data on the phosphatase concentration in pooled milk have also been published *(4, 9)*.

This paper contributes further information on variations in phosphatase activity in raw milk, both from individual cows and from commercial storage tanks. The conditions employed for enzyme assay were comparable to those of the Sanders and Sager *(8)* phosphatase test as accepted by the Association of Official Agricultural Chemists *(2)*.

METHODS

Milks of individual cows were representative of the morning milkings of Holstein and Jersey cows in the University herd and were assayed for phosphatase within 4 hours after cooling to 5° C. Pooled raw milks were obtained

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[1] The data are part of a thesis presented by the senior author in partial fulfillment of the requirements for the degree of Master of Science at the University of Alberta.

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at a local milk plant from 1,500-gal. lots representative of the milk of approximately 500 cows. These samples were assayed for phosphatase within 24 hours after cooling to 5° C. Enzyme activity does not change during such storage.

The procedure of the Sanders and Sager phosphatase test for quantitative results with raw milk was modified because it does not provide an accurate determination of the enzyme *(4, 7)*. Preliminary investigation had shown that relatively greater enzyme activity could be obtained by shortening the substrate hydrolysis period and simplifying the dilution procedure. In the method adopted the reagents used were those of the Sanders and Sager test, but hydrolysis of the substrate (disodium phenyl phosphate) was terminated at the end of 30 minutes. When filtration was completed, 0.50 ml. of clear filtrate was pipetted into a tube containing 9.5 ml. of color development buffer. Four drops (0.08 ml.) of 2,6-dibromoquinonechloroimine (B.Q.C.) solution were added. Then 1.0 ml. of the mixture was diluted to 10.0 ml. in a colorimeter tube with color development buffer. Two more drops of B.Q.C. were added and the color was developed at 25° C. for 30 minutes. Per cent transmittance was read at approximately 600 mμ with an Evelyn photoelectric colorimeter.

The amount of phenol liberated by the enzyme was read directly from a standard curve prepared from aqueous phenol standards. Corrections were made for the dilution and reduced hydrolysis time to make the results comparable to data obtained by the Sanders and Sager method. Phosphatase activity is expressed as micrograms of phenol (equivalent to phenol equivalents) freed by the enzyme in 0.5 ml. of milk during 1 hour.

RESULTS AND DISCUSSION

Milks from individual cows. The effect on phosphatase concentration of milk yield, fat percentage, and breed was studied with milk samples taken weekly from 10 cows during a 14-week period. All of the cows but one (No. 7) had calved during the 8-week period immediately preceding the experiment. Table 1 shows the variation in phosphatase activity and the correlation between phosphatase and weights of the milk. The coefficients of correlation for cows 1, 4,

TABLE 1

Phosphatase activity of milk samples taken at weekly intervals during a 14-week period

Cow		Phosphatase activity		
No.	Breed	Mean	Standard deviation	Correlation with yield
		(γ/0.5 ml.)	(γ/0.5 ml.)	(r)
2	Holstein	444	250	−0.594
3	Holstein	436	231	−0.061
5	Holstein	247	176	−0.820
7	Holstein	688	283	−0.257
8	Holstein	640	170	−0.862
9	Holstein	277	123	−0.824
1	Jersey	390	201	−0.796
4	Jersey	526	367	−0.752
6	Jersey	866	343	−0.881
10	Jersey	361	135	−0.530

5, 6, 8, and 9 are significant at less than the 1% level. These data agree with the results of Folley and Kay, who reported an inverse relationship between milk yield and phosphatase concentration during lactation. In the present study no correlation was found between phosphatase activity and fat percentage or breed. Unusual increases in phosphatase values were observed approximately 3 to 5 weeks after conception in samples from four of the cows. These increases may possibly be related to physiological disturbances associated with pregnancy.

To determine the extent of day-to-day variations, phosphatase activity was determined daily in the colostrum or milk of each of four cows during the first 30 days after parturition. In Figure 1, representative of the results obtained,

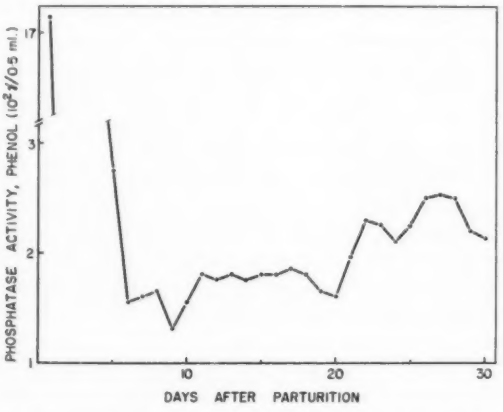

FIG. 1. Phosphatase activity of colostrum and milk samples taken daily from an individual cow (Holstein) during the first 30 days after parturition.

are presented the data for cow 2 (Holstein). Phosphatase values decreased sharply after the first milking, reached a minimum before the tenth day, and then began to increase gradually. These results are consistent with the observations of Folley and Kay except that they found a minimum at 15-25 days after parturition.

The milk from a few cows was assayed to establish phosphatase activity precisely during the complete lactation period. The four cows chosen calved within a 2-week period and were kept under identical conditions. Figures 2 and 3 indicate that the phosphatase trends were similar during the first half of lactation but varied during the later stages. Fluctuations in milk yield did not correspond. The phosphatase curves have maxima approximately 28 weeks after parturition, and the curve for cow 3 shows a sharp rise toward the end of lactation. The change from pasture to winter feeding did not affect the phosphatase trends. Activity determinations ranged from 119 to 4,380 γ phenol, and mean values for cows 1 through 4, were 1,431, 1,191, 1,541, and 2,385, respectively. The coefficients of correlation between phosphatase concentration and milk yield were –0.911, –0.862, –0.700, and –0.902.

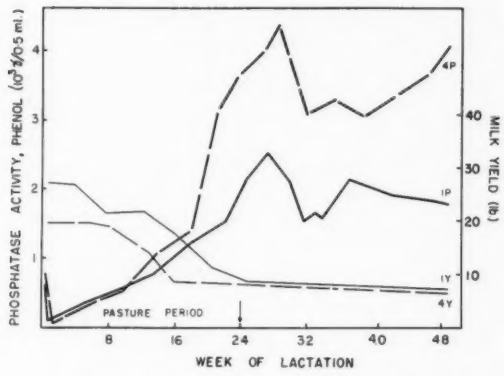

FIG. 2. Phosphatase activity and yield of morning milk at weekly intervals during lactation of Jersey cows No. 1 and 4. 1 P and 4 P — phosphatase determinations, 1 Y and 4 Y — milk yields.

These data show that the milk of individual cows varies widely in phosphatase activity during the course of lactation. Although cows kept under similar management and environmental conditions may exhibit similar trends, the levels of the enzyme in the individual milks vary considerably during the second half of lactation. The results further support Folley and Kay's conclusion that, in general, phosphatase concentration per unit volume of milk increases as milk production declines. It seems unlikely that changes in feed result in immediate fluctuations in enzyme activity during lactation. Kannan and Basu have postulated that fluctuations in the phosphatase content of milk are related to disturbances in the physiological condition of the cows, *e.g.*, changes occurring in the first few days after parturition or during advanced gestation. The

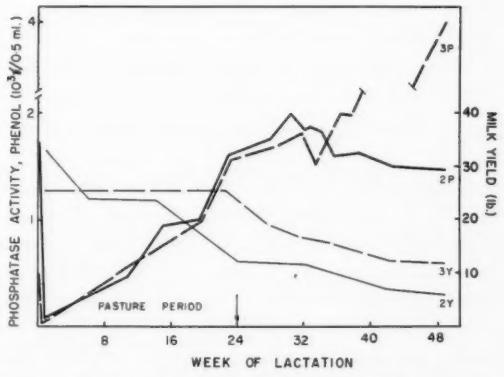

FIG. 3. Phosphatase activity and yield of morning milk at weekly intervals during lactation of Holstein cows No. 2 and 3. 2 P and 3 P — phosphatase determinations, 2 Y and 3 Y — milk yields.

data of the present study are consistent with this theory but are too limited to establish it.

An index to the total amount of phosphatase produced by each cow in a unit of time was obtained by multiplying the enzyme concentration by the weight of milk obtained at the milking at which the sample was taken. When these indices were plotted for the lactation period, the maximum phosphatase output was shown to occur approximately 24 weeks after parturition. This is in contrast to the maximum daily production of milk, which is normally reached around the end of the first month of lactation. Folley and Kay have postulated that a higher rate of phosphatase elimination is caused by a relatively lower level of cellular efficiency in the mammary gland and that the decrease in phosphatase output toward the end of lactation is the result of a decrease in the effective volume of secretory tissue.

Pooled milks. To investigate the extent of seasonal variations, phosphatase activity of pooled milk was determined at weekly intervals. For 4 months, samples were obtained from three milk plants, but there was so little variation between the milks at any one time that determinations were continued for only one plant. Figure 4 shows the data obtained during the period—June, 1953, to May, 1954.

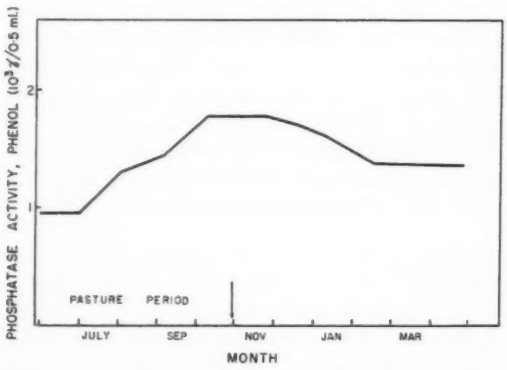

FIG. 4. Phosphatase activity of milk samples taken at weekly intervals from 1,500-gal. lots.

The broad maximum during October, November, and December corresponds to the peaks exhibited by individual cows in Figures 2 and 3. A majority of the cows in the Edmonton region calve in the spring and reach the stage of maximum milk phosphatase activity in the early winter months. Concentration of the enzyme did not change when the cows were removed from pasture. Other factors, such as environmental temperature and plane of nutrition, may be partly responsible for seasonal differences, but the relative effect of these variables cannot be evaluated from the data.

Phosphatase values ranged from 950 to 1,700 γ phenol, with an average of 1,400, and are somewhat higher than those obtained by Hetrick and Tracy, for

ten lots of raw milk, and by Sanders and Sager *(9)*. When pooled milk is derived from a comparatively small number of cows, especially if they calve during the same season of the year, a wider range of variation would be expected than that found in the present study.

The data (Figure 2 and Table 2) of Hetrick and Tracy *(4)* indicate that the rate of inactivation curves for phosphatase determined at 143°, 150°, 155°, and 160° F. are straight lines, except for possible curvature at levels of residual enzyme activity below 4 p.p.m. phenol. Thus one can estimate the differences in time necessary at 143° or 161° F. to reduce different initial phosphatase concentrations to the Sanders and Sager pasteurization standard *(8)*. If a holding time of 30 minutes at 143° or of 15 seconds at 161° F. is required to decrease a phosphatase value of 950 γ phenol per 0.5 ml. of milk to 2 γ, then an initial value of 1700 γ will require 32.75 minutes at 143° or 16.4 seconds at 161° F. (using the same pasteurizers). The importance of the initial concentration of phosphatase is substantiated by the fact that cream requires a more severe heat treatment to yield a negative phosphatase test than does milk *(4, 10)*. It is possible that at certain seasons of the year pooled milk might be encountered that contains sufficient phosphatase to yield a positive phosphatase test after being subjected to the minimum heating conditions for pasteurization *(11)*.

SUMMARY

The phosphatase activity of milk, determined during one complete lactation period of individual cows, was found to vary from 119 to 4,380 γ phenol per 0.5 ml. of milk. Minimum and maximum values occurred approximately 1 week and 28 weeks after parturition, respectively. Enzyme concentration was related inversely to milk yield, but no correlation was observed between phosphatase and breed, fat percentage, or feed. Maximum total phosphatase production per milking was reached approximately 24 weeks after parturition.

During a complete year the phosphatase activity of pooled milks, representative of approximately 500 cows, ranged from 950 to 1,700 γ phenol per 0.5 ml. of milk. The trend corresponded to that observed during the lactation of individual cows, with maximum values occurring in early winter. Variations in initial phosphatase activity could influence the heat treatment required to inactivate the enzyme to the Sanders and Sager pasteurization standard.

ACKNOWLEDGMENTS

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PRECIPITATION OF CALCIUM CASEINATE BY HEAT AND SUBSEQUENT REVERSAL

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The precipitation of calcium caseinate by heat (90° C. for 30 to 60 minutes) in a range of pH values and calcium concentrations was measured. Changes in viscosity and clarity of the heated solutions were determined. The effect of cream on the re-solution of the casein precipitates is described. *Editor.*

A study of the effect of heat on calcium caseinate might be expected to contribute to an understanding of the physical changes in heated milk, since casein is a major component of milk. The relatively few studies of this type that have been done have been limited in scope and will be discussed later. In the present investigation the precipitation of calcium caseinate by heat (90° C. for 30 to 60 minutes) in a range of pH values and calcium concentrations has been measured. Changes in viscosity and clarity of the heated solutions have also been determined. Subsequent re-solution of the precipitates at 25° C. has been observed and the extent of this re-solution measured. The effect of cream on the re-solution of the casein precipitates is described.

MATERIALS AND METHODS

Casein. The casein was precipitated from skimmilk by acidification to pH 4.5 with *N* HCl. The precipitate was washed four times with water and twice dissolved and reprecipitated with acid *(2)*. The casein was finally dried with ethanol and ether. The casein was stored in a desiccator at a relative humidity that maintained the moisture content at 10.0%. The concentration given in the results are for the moisture-free product.

Calcium hydroxide. A standard solution of calcium hydroxide was prepared at 20 to 25° C. The concentration was determined by titration with standard HCl and found to be 0.045 *N*.

Preparation of casein solutions. The casein solutions were prepared and used within 1 to 3 days. When not in use the solutions were stored at 7° C. A stock 4% sodium caseinate solution at pH 6.8 was prepared by dissolving 1.0 g. (moisture-free) of the isoelectric casein in water, with 5.8 ml. of 0.1 *N* NaOH, in a final volume of 25 ml. A 4% calcium caseinate solution at pH 7.0 was prepared

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[1] A laboratory of the Eastern Utilization Research Branch, Agricultural Research Service, USDA.

by dissolving 1.0 g. of casein in water, with 15.0 ml. of 0.045 *N* $Ca(OH)_2$, in a final volume of 25 ml. The concentration of calcium in this solution was 0.0135 *M*. Other pH values were obtained by the addition of more or less NaOH or by the addition of HCl. The sodium caseinate–calcium chloride solution is considered approximately equivalent to calcium caseinate. The binding of the calcium ion to casein, however, will be lessened somewhat by the presence of sodium ions (unpublished studies).

Heating. Four milliliters of the solution to be tested was placed in a 12-ml. Pyrex centrifuge tube. A smaller tube containing ice water was suspended within the centrifuge tube to prevent loss of water by evaporation. The solutions were heated for various periods at 90° C. in a constant level water bath. The pH values were measured before and after heating; in most cases the values were identical. After heating, the solutions were immediately brought to 25° C. by placing the tubes in water at that temperature.

Centrifuging and sampling. The heated samples after variable periods of time (2 minutes to 3 hours) at 25° C. were centrifuged for 10 minutes at 3,000 × G. The top 0.5 ml. of the solution was withdrawn for analysis, taking care not to disturb the remainder of the solution. The amount of sample taken was kept constant since when the precipitates are colloidal a concentration gradient was apparent after centrifuging.

Determination of casein. (a) The casein in the supernatant fluid from the centrifuged samples in some instances was estimated from the nitrogen content, determined by the Kjeldahl procedure. *(b)* The casein was also estimated from the light absorption at 280 mμ. The heated solutions were clarified by mixing 0.15 ml. of 0.1 *M* disodium versenate, adjusted to pH 8.0 with 0.1 *N* NaOH, with 0.5 ml. of the calcium caseinate solution. Water was added, usually to a final volume of 25 ml., to give a dilution of the casein solution of 1:50, for the light absorption measurements at 280 mμ. An absorbance of 0.85 in a 1-cm. cell is equivalent to 1.0 mg. of whole casein per 1.0 ml. The absorbance factor for α-casein is 1.03 and for β-casein is 0.48 *(2)*. Hence if a fractionation of the casein occurred in the heat precipitation, the protein estimations would be somewhat in error. There is, however, no evidence that a separation of α- and β-casein occurs.

Viscosity and opacity determination. The viscosity was determined at 30° C. in a Bingham type viscometer with a variable pressure head. For this measurement a volume of 6.0 ml. was heated in a 1-inch diameter test tube. The opacity or whiteness of these solutions was estimated from the apparent light absorption at 600 mμ. Details of the viscosity and opacity measurements are described in a previous paper *(7)*.

RESULTS

Effect of calcium concentration on viscosity of unheated and heated sodium and calcium caseinates at pH 7.0. The effect of calcium chloride concentration on the viscosity of 2.0% solutions of sodium and calcium caseinates at pH 7.0 is shown in Figure 1. The calcium caseinate curves (unheated and heated) are

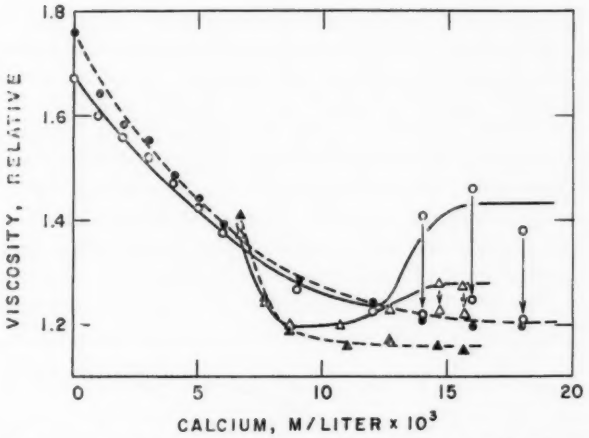

FIG. 1. The viscosities of 2% sodium and calcium caseinate solutions at pH 7.0 with increasing calcium concentrations, before heating and after heating for 30 minutes at 90° C. and cooling. Calcium was added to both caseinates as calcium chloride. Calcium concentration given is total calcium.

Sodium caseinate: unheated ●-----●; heated ○——○
Calcium caseinate: unheated ▲-----▲; heated △——△
The vertical arrows indicate the drop in viscosity of the heated solutions after 1 hour at 30° C.

plotted in terms of the total calcium concentration, that is, the graphs begin with the calcium concentration (0.0067 *M*) of the calcium caseinate solution.

Small concentrations of calcium decrease the viscosity of the casein solutions, but at total concentrations of calcium above 0.012 *M*, the viscosities of both heated sodium and heated calcium caseinates are greater than their viscosities before heating. This increase in viscosity is largely reversible and is due to the formation of precipitates of colloidal calcium caseinate which redissolve at lower temperatures.

The appearance of these solutions is of interest since opacity or light scattering (measured by apparent absorbance at 600 mμ) is indicative of aggregation. The unheated sodium caseinate remains clear (absorbance of 0.05 or less) with concentration of calcium chloride up to 0.005 *M;* with greater concentrations of calcium an opalescence appears, which increases considerably with time even at 25° C. For calcium concentrations of 6, 8, 10, 12 and 14×10^{-3} *M*/l., the respective absorbance readings at 25° C. within a few minutes of adding the calcium chloride are 0.15, 0.34, 0.59, 0.94 and > 2. The light scattering, that is the aggregation, is greatly reduced if the solutions are cooled below 25° C. As one might expect, the light scattering is increased by heating; this is quickly reversible to a considerable extent when the solutions are lowered to 25° C. With 0.005 *M* calcium the reversal is complete: before heating, the absorbance is 0.05; at 90° C. the solution is opaque (absorbance > 2); when returned to 25° C., the absorbance is 0.15, and within 1 hour it is at its original value of 0.05. With

0.006 *M* calcium or greater absorbance is > 2 at 90° C.; at 25° C. the absorbance drops (to 0.3 with 0.006 *M* calcium) and subsequently increases again.

Precipitation of casein by calcium chloride and heat (90° C.) and re-solution at 25° C. Relatively large concentrations of calcium chloride are required to precipitate sodium caseinate as the calcium salt without heat; the extent is partially pH-dependent. For example 0.02 *M* calcium chloride in a 2.0% sodium caseinate solution precipitates 30% at pH 5.6, 20% at pH 6.0 and 6.6, and 8% at pH 7.6. In the calcium chloride range of 0.015 to 0.025 *M* the extent of precipitation is proportional at each pH to the concentration of calcium; for example, precipitation at pH 5.6 declines to 12% at 0.015 *M* calcium chloride concentration. The precipitation of sodium caseinate by calcium chloride is in-

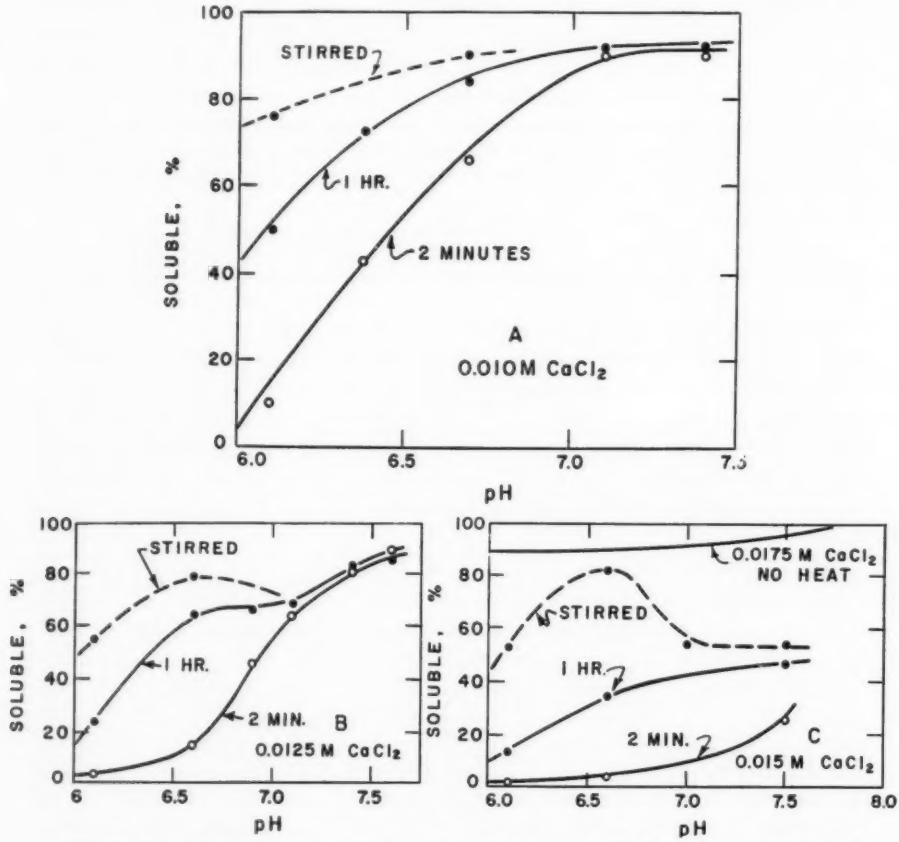

FIG. 2. Precipitation of 2% sodium caseinate solutions at pH 6.1 to 7.6 containing calcium chloride after heating for 1 hour at 90° C. The lower solid curves show the portion remaining in solution immediately after cooling to 25° C. (about 2 minutes required), and the upper curves the portion in solution 1 hour after bringing to 25° C. The dashed lines indicate the solubility attained when the precipitates are stirred every 10 minutes for 1 hour.

creased by heating at 90° C. Much of this precipitation reverses at 25° C., but in no case did the solubility return to the value before heating. The effect of pH and calcium concentration on this heat-produced precipitation is shown in Figures 2-A to -C. Similar experiments were also done with 0.0075 *M* $CaCl_2$, a concentration that gave a precipitate (80% remained soluble) only at pH 6.1. The percentage in solution is shown for 2 minutes (time required to cool to 25° C. and begin centrifugation) and 1 hour after heating. The values plotted are averages of 2 to 10 or more experiments. In a few instances the reversed (1 hour) values have been as much as 20% higher than the values shown. The occasional high value may be due to difficulty in centrifuging and sampling these colloidal precipitates. These data (shown by solid lines) were obtained with precipitates that were undisturbed during the 25° C. period. Much of the precipitate adheres to the walls of the tubes when the precipitates are flocculent, as with high calcium concentration and low pH. It was found, however, that if the precipitates were broken up and kept in suspension, a higher level of solubility was attained on standing at 25° C. For example, at pH 6.1 and 0.0125 *M* calcium chloride almost all of the casein was precipitated on heating; after 1 hour at 25° C. undisturbed, about 20% of the total casein was in solution, and this amount had not increased in 2 hours. If, however, the precipitate was broken up and kept in suspension, 55% was in solution in 1 hour and 59% in 2 hours. Data obtained at other pH and calcium values when the precipitates are kept suspended are shown in Figures 2-A to -C by dashed lines. The precipitation of the casein by heat at pH 6.1 is plotted in Figure 3 with the calcium concentration as the ordinate to emphasize the influence of this important variable. Also shown is the solubility curve of the unheated casein solution.

Factors other than pH and calcium concentration also influence the precipitation and re-solution of the precipitates. The influence of the time of heating on the amount of precipitate at pH 6.6 and 0.0125 *M* calcium chloride is

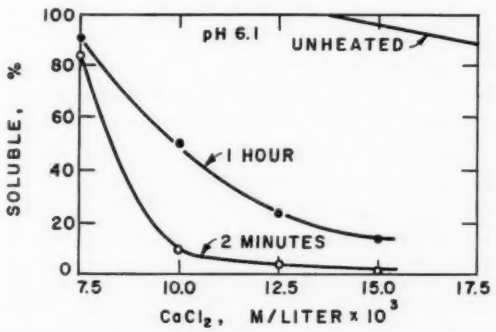

FIG. 3. Precipitation of 2% sodium caseinate solutions containing 0.0075 to 0.0175 *M* calcium chloride at pH 6.1 after heating for 1 hour at 90° C. The lower curve shows the portion remaining in solution immediately after cooling to 25° C. (about 2 minutes required) and the curve above this the portion in solution 1 hour after cooling to 25° C. The short upper right curve indicates the solubility of the unheated solution.

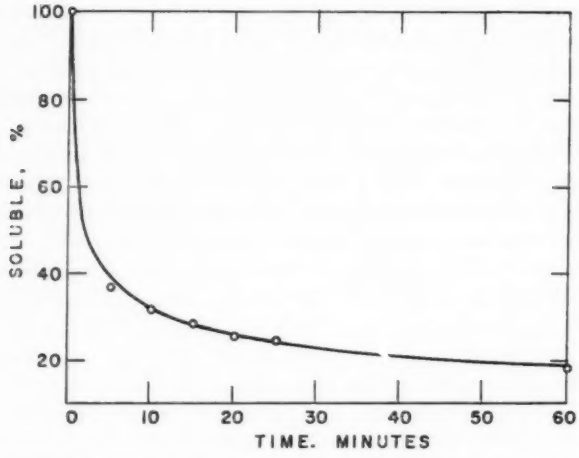

FIG. 4. Precipitation of 2% casein containing 0.0125 *M* calcium chloride at pH 6.5 after heating at 90° C. for various periods of time and bringing to 25° C.

shown in Figure 4. The appearance of the precipitate also changes during the heating period. The initial precipitates are colloidal and become more flocculent and dense as the heating continues. A comparison of solutions at this pH and calcium concentration heated for 10 minutes and 60 minutes indicated that the rate of re-solution of the precipitates was about the same but the solution heated for 10 minutes reached a higher level of solubility at 25° C. Heated solutions permitted to stand at 25° C. took about 1 hour for re-solution. Heated solutions standing at 3° C. redissolved more slowly but about the same solubility level was attained. A comparison of the rate of re-solution at the two temperatures is

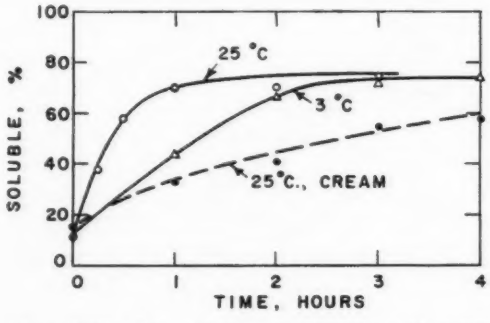

FIG. 5. The rate of re-solution of heated casein precipitates at 3° and 25° C., and the effect of cream at the latter temperature. The 2% casein solutions were at pH 6.5, contained 0.0125 *M* calcium chloride, and were heated for 1 hour at 90° C. The total casein value for the cream experiment was determined with the cream present on a centrifuged sample before heating. Cream (approximately 25% butterfat) was added to 2% by volume.

shown in Figure 5. This figure also contains data illustrating the influence of cream on the re-solution of the calcium-casein precipitates.

The effect of cream on the heated casein solutions was studied to see whether the re-solution of the precipitates would be hindered, perhaps by their being physically coated with the cream. The results in Figure 5 show that this actually occurs. It was anticipated that parallel changes in viscosity would occur. The viscosity measurements, however, were unsatisfactory for showing such an effect. A 2% solution of sodium caseinate containing no cream, at pH 6.6 and with 0.0125 *M* calcium chloride, had a relative viscosity of 1.22 before heating and 1.42 after heating, and within 30 to 50 minutes the viscosity had declined to 1.22. The same system containing 2 or 10% cream (approximately 25% butterfat) had an initial viscosity of 1.45, which did not increase when the mixture was heated. Apparently, although the casein has precipitated, parallel changes in the physical state of the cream prevent a change in the viscosity.

DISCUSSION

The reduction in viscosity of 2% calcium caseinate, pH 7.0, on the addition of calcium chloride confirms almost exactly results of Hankinson and Briggs *(1)* at pH 6.6. These authors, however, reported that sodium caseinate showed structural viscosity, and this has not been observed in the present studies. Viscosity measurements on 1% sodium caseinate with several concentrations of sodium chloride were only 1% lower than the data of Hankinson and Briggs, although these authors stated that their measurements were made with a pressure of 30 cm. of mercury to eliminate pressure dependence. Heating the sodium caseinate to 90° C. did not induce structural viscosity. Examination of different preparations of casein would be desirable to explain these divergent results.

The reduction in the viscosity of caseinate solutions on the addition of NaCl or $CaCl_2$ in part is due to the reduction in electroviscosity *(1)*. It is apparent, however, that the calcium ion has also a specific effect, as shown by its ability to aggregate casein, since the opacity of the solutions increased with the addition of calcium. This aggregation may also contribute to the reduction in viscosity, perhaps by the formation of more compact and symmetrical aggregates, which might explain the fact that calcium caseinate is considerably less viscous than sodium caseinate. The ability of the calcium ion both to aggregate and to reduce viscosity was observed in heated β-lactoglobulin solutions *(7)*, and aggregation of β-lactoglobulin by heat near the isoelectric point leads to a reduction in viscosity (unpublished studies).

The increase in viscosity on heating casein solutions containing more than 0.012 *M* calcium is due to the formation of a colloidal casein precipitate. When the calcium concentration is high enough, however, to give a precipitate of discrete floccules, viscosity measurements are lower and are, of course, meaningless. The precipitation of casein is greatest at high calcium concentration and low pH values, as was observed for β-lactoglobulin *(7)*. With casein, however, even

at concentrations of calcium that almost totally precipitate the casein at 90° C., much of the precipitate will go back into solution at 25° C. The marked effect of stirring on the re-solution of the precipitates at pH 6.6 is not clear. It was expected that the unstirred precipitates would attain the same solubility as the stirred samples, but more slowly. Cessation of re-solution of the unstirred precipitate between 1 and 2 hours suggests that some factor, perhaps a coalesced film of precipitate, is hindering further solution.

The re-solution of calcium caseinate precipitates obtained with heat apparently has not hitherto been reported. Pyne *(5)* has observed that low calcium concentrations precipitated β-casein and that the precipitate redissolved on cooling. Although this appears to be an unusual solubility property of calcium caseinate, this increase in solubility at low temperature is characteristic of calcium phosphate and of some other inorganic salts. This behavior of inorganic salts is thought to be due to the formation of soluble hydrate at low temperatures and of less soluble anhydride at high temperature. Something simliar may be true of calcium caseinate, perhaps through hydration at the phosphate groups.

The precipitation of casein by heat has been investigated by Howat and Wright *(3)*. The treatment used was severe, up to 5 hours at 120° C. The precipitation of calcium caseinate (3.0% concentration at pH 6.5 to 7.0) at 120° C. was less than 10% in 1 hour. In 3 to 5 hours precipitation was about 90%, but under this severe treatment 80% of the phosphate was liberated from casein. The heat treatment used in the present studies (90° C. for 1 to 3 hours) does not precipitate calcium caseinate unless additional calcium chloride is added. This heat treatment will release little or no phosphate from casein *(4)*, and it appears that heat processing of milk is not so severe that phosphate will be released from casein. Howat and Wright *(3)* found that only calcium caseinate precipitated, although both calcium and sodium caseinates released phosphate on heating. These authors also observed the enhanced precipitation of casein with additional calcium; if the calcium content of the calcium caseinate was increased 16% with calcium chloride, the precipitation in 1 hour at 120° C. was now about 30%.

The ability of cream to slow up the re-solution of the heated calcium caseinate precipitates may be of significance in the storage of heat-processed milk. The drop in viscosity is reminiscent of the drop in viscosity on storage of evaporated milk *(6)*, although the latter takes several weeks for completion. The formation of colloidal casein precipitates during sterilization of milk and their slow re-solution on storage may account for the slow drop in viscosity on the storage of evaporated milk. This possibility, however, is made less likely by the finding that the addition of sodium phosphate (unpublished studies) makes the casein precipitates more dense and less colloidal, and retards or prevents re-solution of the precipitates. The slowing of the re-solution of heated casein precipitates by cream probably results from a physical coating of the colloidal calcium caseinate precipitates, which hinders access to the aqueous phase into which the precipitate can dissolve.

SUMMARY

The addition of calcium chloride to 2% sodium or calcium caseinate solutions causes a drop in viscosity, perhaps through aggregation since the solutions become opalescent. If the total concentration of calcium is not greater than 0.012 *M*, heating to 90° C. for 1 hour does not change the viscosity, but the solutions become more opalescent and even opaque (white). The opalescence is completely reversible, at the lower calcium concentrations, when the solutions are cooled. At concentrations of calcium above 0.012 *M* the viscosity is increased by heating to 90° C.; on standing at lower temperatures the viscosity increase is partly reversed in 1 to 3 hours. The viscosity increase is due to the formation of a colloidal precipitate of casein which partly redissolves at lower temperatures. The amount of precipitate is increased by high calcium concentration and low pH values. Other factors that affect the precipitation and re-solution are time of heating, stirring, and temperature after heating. Cream slows up the re-solution of the colloidal casein precipitates.

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INTERRELATIONSHIPS BETWEEN CAROTENE FROM ARTIFICIALLY DEHYDRATED ALFALFA AND VITAMIN A FROM A DRY CARRIER WHEN FED SIMULTANEOUSLY TO HOLSTEIN CALVES[1]

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With the synthesis and commercial production of crystalline vitamin A and its incorporation into a dry carrier, its use by commercial feed manufacturers has greatly increased. However, there is a lack of information on the interrelationships between carotene and vitamin A per se when fed simultaneously. Forty-eight 1-day-old male Holstein calves were used as test animals. Blood samples taken weekly from the 35th to the 175th day of age were analyzed for plasma vitamin A and carotenoid content. At the time of slaughter the liver of each test animal was analyzed for vitamin A and carotenoid content. The practical significance of the study is the finding that both sources of vitamin A may be included simultaneously in a ration without adverse effects. *Editor.*

With the elucidation of the chemical structure of carotene and vitamin A, their interrelationships have become more readily understood. There have, however, been several reports which indicated that the relationship is not as simple as the chemical structures might indicate. Using primarily plasma carotenoids and vitamin A as criteria, several workers have investigated the effect of massive doses of either compound when fed with the other to dairy animals. Large doses of vitamin A have been reported to suppress plasma carotenoid levels both in the calf *(7, 10, 15, 18)* and in older animals *(3, 8, 16, 21, 23)*. A similar suppression of the carotenoids in milk following large doses of vitamin A to milking animals has been observed *(3, 9, 11, 14)*. In none of these studies was the intake

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of both sources of the vitamin controlled simultaneously nor was an attempt made to give quantitative expression to the results.

With the synthesis and commercial production of crystalline vitamin A and its incorporation into a dry carrier, its use by feed manufacturers has increased considerably. In addition, it is frequently used in combination with artificially dehydrated alfalfa, which serves as a source of carotene. In view of the lack of quantitative information on the interrelationships between carotene and vitamin A per se when fed simultaneously and the need for such information under current feeding practices this experiment was undertaken.

EXPERIMENTAL PROCEDURE

Animals and rations. Forty-eight 1-day-old male Holstein calves were placed on experiment during the period October, 1953, to January, 1954. These calves were obtained from various Connecticut institution herds and placed in individual tie stalls at the University research barn. Upon arrival at the research barn each calf received a 500-mg. oblet of chlortetracycline (aureomycin)[3] and 200,000 U.S.P. units of vitamin A in the form of fish liver oil.[4]

The animals were raised to 35 days of age on a limited whole milk *(4)*, limited dry calf starter *(6)*, and ad libitum hay regime. The hay was U.S. No. 1 alfalfa, chopped at a setting of ¾ inch. From the 35th to the 63rd day of age, the initial depletion period, the animals were fed a vitamin A depletion ration *(6)*, the allowance of which was calculated from the formula $y = 0.0562\ w^{0.87}$ in which y is the daily depletion ration allowance in pounds and w is the anticipated live weight based on an estimated 10 lb. weekly increase. This depletion ration allowance was continued until the animals were slaughtered at 175 days of age.

When the animals were 63 days of age, the depletion ration was supplemented simultaneously with one of four levels of carotene: 20, 50, 125, or 312.5 γ per pound of live weight daily, and one of four levels of vitamin A: 4, 10, 25, or 62.5 γ per pound of live weight daily. The carotene was supplied as artificially dehydrated alfalfa leaf meal[5] and the vitamin A from a dry carrier.[6] The first 16 calves to arrive at the research barn were randomly assigned to one of the 16 supplement combinations, and this procedure was repeated until three replicates of 16 calves each had been assigned to their respective treatments. The carotene and vitamin A supplements were fed for a 12-week period, 63 to 147 days of age, defined as the comparison period. Thereafter the animals received the unsupplemented depletion ration for an additional 4 weeks, 147-175 days of age, defined

[3] The aureomycin oblets were supplied by the Lederle Laboratories, Pearl River, N.Y., through the courtesy of Dr. Ralph Elliott.

[4] The fish liver oil contained 25% by weight of crude soybean lecithin and 25,000 U.S.P. units of vitamin A per gram. It was supplied by the Nopco Chemical Co., Harrison, N.J., through the courtesy of Dr. Melvin Hochberg.

[5] The artificially dehydrated alfalfa leaf meal was obtained from the W. J. Small Co., Div. of Archer-Daniels-Midland Co., Kansas City, Mo. It contained no additives such as antioxidants.

[6] The vitamin A was in the dry stabilized form, Nopcay 10 Type IV and was supplied by the Nopco Chemical Co., Harrison, N.J., through the courtesy of Dr. Melvin Hochberg.

as the final depletion period, in order that body stores of vitamin A might be estimated. The procedure for calculating and incorporating the carotene and vitamin A supplements into the depletion ration was as previously described *(18)*.

The calves were exposed only to artificial light from 6 A.M. to 6 P.M. daily. The average intensity of light at a height of 48 in. in the center of each calf stall was 6.2 foot candles[7] with a standard deviation of 4.1. The average daily minimum and maximum temperatures in the calf unit during the supplement feeding period was $54 \pm 7°$ F. and $68 \pm 7°$ F., respectively. Standard procedures followed at this station *(1)* were used in the treatment of diarrhea.

Observations and analyses. All feeds fed and refused were weighed to the nearest 0.1 lb. Live weights were taken upon arrival at the barn, at 7 days of age and at 7-day age intervals thereafter to the 175th day of age. Height at withers, heart girth, and girth of paunch were measured to the nearest $\frac{1}{4}$ in. on the 63rd day of age and at 28-day intervals thereafter to the 175th day of age.

Blood samples, taken by jugular puncture, were obtained at 7-day intervals from the 35th through the 175th day of age and analyzed for plasma vitamin A and carotenoid content. At the time of slaughter the entire liver was removed, ground, and analyzed for vitamin A and carotenoid content.

All feeds fed and refused were sampled and analyzed at 28-day intervals throughout the entire experimental period for proximate constituents and carotene content. The carotene and vitamin A supplements were sampled and analyzed for carotene and vitamin A content at 14-day intervals throughout the supplement feeding period. In addition, each new 50 lb. bag of alfalfa leaf meal was sampled immediately prior to its being fed, as was each new 1 lb. jar of the vitamin A concentrate. The analyses for proximate constituents and for carotene and vitamin A in the feeds and the supplements fed and for the carotenoids and vitamin A in blood plasma and liver were as previously described *(18)*. The results of the feed analyses are presented in Table 1. The alfalfa leaf meal contained 0.225 ± 0.021 mg. carotene per gram and the vitamin A content of the dry carrier expressed as vitamin A alcohol was 3.57 ± 0.04 mg. per gram.

One calf died at 2 months of age, which was prior to its supplement feeding period when it would have received 4 γ of vitamin A and 50 γ of carotene per pound of live weight. Death was attributed to pathological lesions of the liver and kidney, possibly due to a severe enteritis and prolonged sulfonamide treatment. The missing values for the various criteria were estimated according to standard procedures. Owing to a few weighbacks of the depletion ration containing the supplements during the first 4 weeks of the comparison period, only the data from the last 8 weeks of this period, 91 to 147 days of age, were utilized. Standard statistical procedures were employed in the analysis of the data *(2, 20)*.

Depletion time, the number of weeks required for plasma vitamin A values to decrease to 4 γ per 100 ml. of plasma, was used as an estimate of body stores of vitamin A. Depletion time was calculated according to the method of Teichman

[7] Measured with a Weston foot-candle meter, Model 614 by H. C. Cornish of the Conn. Light and Power Co., Willimantic, Conn.

TABLE 1
Average chemical composition of feeds

	Per cent dry matter	Per cent of dry matter					
		Crude protein	Ether extract	Crude fiber	N.F.E.	Ash	Carotene (mg/lb.)
Alfalfa hay—chopped	92.44 ± 0.42	17.93 ± 0.52	2.22 ± 0.05	25.99 ± 1.82	45.64 ± 1.13	8.23 ± 0.33	20.89 ± 1.36
Calf starter	91.72 ± 0.32	22.43 ± 0.33	3.81 ± 0.32	7.32 ± 1.68	59.53 ± 1.78	6.91 ± 0.12	0.27 ± 0.06
Vitamin A depletion mixture	89.63 ± 0.68	15.93 ± 0.21	2.86 ± 0.20	13.35 ± 0.36	61.37 ± 0.41	6.48 ± 0.10	0.09 ± 0.01
Artificially dehydrated alfalfa leaf meal	93.24 ± 0.41	24.76 ± 0.67	3.83 ± 0.09	17.19 ± 0.88	40.62 ± 0.49	13.59 ± 0.47	101.96 ± 9.69
Dry stabilized vitamin A supplement	94.58 ± 0.26	31.29 ± 1.61	30.87 ± 2.07	4.22 ± 0.20	29.23 ± 1.62	4.39 ± 0.16	—

et al. (22) by utilizing the rate of decrease of plasma vitamin A values during the final 4-week depletion period, 154-175 days of age. During the last week of the final depletion period, 168-175 days of age, spinal fluid pressures were measured on each calf according to the method of Moore *et al. (13)*.

RESULTS AND DISCUSSION

Feed consumption, growth, and health. With the exception of the first four weeks of supplement feeding, the comparison period, calves readily consumed their respective ration allowances. At the beginning of the comparison period, 63 days of age, the average calf weighed 163 ± 17 lb. and measured 33.0 ± 1.2 in. in height at withers, 35.7 ± 1.7 in. in heart girth, and 43.3 ± 2.0 in. in girth of paunch. Increases in these measurements, from 91 to 147 days of age, the last 8 weeks of the comparison period, were, respectively, 103 ± 11 lb., 3.9 ± 0.5 in., 5.6 ± 0.8 in., and 6.8 ± 1.4 in. These criteria, in general, were unrelated to carotene and/or vitamin A intake. The average calf had less than one day with diarrhea during the comparison period. This criterion was found to be unrelated to the level of carotene and/or vitamin A feeding.

Blood and liver. The average plasma values during the last 8 weeks of the comparison period, 91–147 days of age, are presented in Table 2. For reasons previously discussed *(5)*, logarithms of the plasma values were examined with respect to their response to the log-log (carotene intake) and log-log (vitamin A intake plus ten). The logarithms of both plasma carotenoids and plasma vitamin A were found to increase in an essentially linear manner with increases in carotene intake expressed as log-log. Similarly the logarithms of plasma vitamin A increased in a linear manner with increases in log-log (vitamin A intake plus ten), but as previously reported *(7, 10, 15, 18)* the logarithms of plasma carotenoids decreased with increasing intakes of vitamin A. This decrease of the logarithms of plasma carotenoids occurred in a linear manner with increases in the log-log (vitamin A intake plus ten). Since no significant interaction was found, it can

TABLE 2

Response of plasma vitamin A and plasma carotenoids to the simultaneous feeding of various levels of carotene and vitamin A

Carotene intake	Vitamin A intake	Plasma carotenoids			Plasma vitamin A			
		Initial[a]	Comparison[b]		Initial[a]	Comparison[b]		
			Actual	Log		Actual	Log	Adjusted log[c]
(γ/lb. live weight/day)					*(γ/100 ml. plasma)*			
20	4	4	10	0.980	14.2	15.6	1.181	1.172
	10	6	8	0.877	13.6	22.3	1.346	1.341
	25	7	8	0.853	12.6	29.2	1.464	1.464
	62.5	10	6	0.730	12.4	26.4	1.415	1.416
50	4	7	25	1.378	13.0	20.0	1.298	1.295
	10	7	25	1.388	9.8	21.1	1.314	1.329
	25	5	10	0.990	11.1	27.6	1.435	1.443
	62.5	3	13	1.080	12.9	29.2	1.464	1.463
125	4	15	49	1.683	15.4	24.1	1.374	1.359
	10	4	42	1.590	12.1	25.5	1.394	1.397
	25	5	37	1.559	13.5	30.1	1.470	1.465
	62.5	12	30	1.438	8.2	28.6	1.455	1.478
312.5	4	10	104	2.009	11.2	23.9	1.375	1.383
	10	5	89	1.941	8.9	25.2	1.396	1.416
	25	10	85	1.918	13.8	28.0	1.432	1.426
	62.5	9	87	1.924	18.5	33.5	1.517	1.486
Standard deviation of a calf		3	—	0.115	4.5	—	0.066	0.064

[a] Av. value for initial depletion period 35 to 63 days of age, 3 calves per group.
[b] Av. value for 91st to 147th day of age, 3 calves per group.
[c] Values of comparison period adjusted for average value of initial depletion period.

be assumed that the responses to carotene and vitamin A feeding were additive with respect to plasma vitamin A. Although the response of plasma carotenoids to carotene and vitamin A feeding was found to be unrelated to initial stores of carotene as estimated by the average plasma value during the initial depletion period, 35-63 days of age, the increases in the logarithms of plasma vitamin A were significantly influenced by initial stores of vitamin A, estimated in a similar manner, and therefore the appropriate adjustments were made. The over-all relationships were best described by the following formulas:

$$\hat{y}_C = 0.550 + 3.807x_1 - 1.060x_2$$

and

$$\hat{y}_A = 1.139 + 0.300x_1 + 0.740x_2 + 0.005x_3$$

where $\hat{y}_C$ and $\hat{y}_A$ are the logarithms of the predicted plasma carotenoids and vitamin A, respectively, expressed as micrograms per 100 ml. plasma, x_1 and x_2 are the log-log (carotene intake) and log-log (vitamin A intake plus ten), respectively, expressed in micrograms per pound of live weight per day, and x_3 is the average plasma vitamin A value during the initial depletion period in micrograms per 100 ml. of plasma.

Statistical analysis of plasma carotenoid and plasma vitamin A values, by weeks, from 98-147 days of age, the comparison period, indicated that these criteria

increased in a linear manner with time, levels of significance being $P < 0.01$ and $P < 0.05$, respectively. Since the rates of increase of the two criteria were constant and were found not to interact with treatments, average values were used in further analyses. In view of the fact that carotene and vitamin A intakes per pound of body weight were held constant throughout the experiment, the increases in the respective criteria may be thought of as reflecting increasing stores of the nutrient, or variations, concomitant with age, in the requirement per pound of body weight, when plasma values are used as criteria of vitamin A status. These implied variations in requirement may also be due to changes in blood–body weight relationships and/or changes in liver weight–body weight relationships *(1)*.

Liver carotenoids, liver vitamin A, and liver weight values are presented in Table 3. It was assumed that liver stores of both carotenoids and vitamin A at 175 days of age, after a 4-week depletion period, would maintain the same relationship between treatments that existed at 147 days of age, which was the last day of the supplement feeding period. The stores would, of course, all be smaller.

TABLE 3

Influence of the simultaneous feeding of various levels of carotene and vitamin A on body stores of carotene and vitamin A and on spinal fluid pressure

Carotene intake	Vitamin A intake	Liver[a]						Predicted depletion time[b]		Spinal fluid pressure[a]	
		Weight	Carotenoids		Vitamin A						
			Actual	Log	Actual	Log	Adjusted log[c]	Actual	Adjusted[c]	Actual	Log
(γ/lb. live weight/day)		*(g.)*	*(γ/100 g. liver)*					*(wk.)*		*(mm. H_2O)*	
20	4	2773	15	1.175	17	1.125	1.092	3.24	2.94	232	2.329
	10	3225	19	1.276	65	1.658	1.626	4.79	4.60	127	2.083
	25	2890	25	1.376	641	2.641	2.641	8.87	8.87	148	2.160
	62.5	2850	14	1.018	3189	3.475	3.481	12.31	12.34	108	2.031
50	4	3013	22	1.537	201	1.518	1.504	4.67	4.59	368	2.578
	10	2744	26	1.412	118	2.063	2.151	6.45	6.95	123	2.080
	25	2654	19	1.271	315	2.492	2.539	10.15	10.42	123	2.082
	62.5	2651	17	1.214	2471	3.318	3.308	13.27	13.21	90	1.954
125	4	3053	35	1.531	126	2.071	1.982	7.00	6.48	123	2.079
	10	2954	35	1.539	376	2.305	2.320	7.42	7.50	103	2.007
	25	2685	48	1.676	978	2.981	2.951	9.98	9.81	117	2.060
	62.5	2715	22	1.261	3498	3.443	3.580	13.55	14.34	132	2.117
312.5	4	3084	73	1.833	700	2.823	2.868	8.60	8.86	123	2.088
	10	2681	62	1.771	665	2.718	2.834	9.57	10.24	113	2.045
	25	3069	59	1.757	1181	2.981	2.943	11.10	10.88	112	2.048
	62.5	2695	48	1.658	5695	3.735	3.546	14.16	13.07	97	1.983
Standard deviation of a calf		224	—	0.195	——	0.313	0.287	1.62	1.44	——	0.061

[a] Values at 175 days of age after 4 weeks of depletion, 3 calves per intake group.
[b] Based on blood plasma values during the 4 weeks of the final depletion period.
[c] Adjusted for average plasma value during initial depletion period, 35-63 days of age, prior to carotene and vitamin A being added to ration.

This assumption was made on the grounds that plasma vitamin A under controlled experimental conditions reflected liver stores *(17)* and that the rate of decrease of plasma vitamin A values when the animals are receiving a vitamin A depletion ration is independent of initial stores as reflected by plasma vitamin A at the beginning of the depletion period *(22)*. For reasons previously explained *(19)* the logarithms of these data were taken and examined with respect to the log of intake. Liver weights were found to be unrelated to treatment.

Liver carotenoids were found to be unrelated to initial liver stores as estimated by the average plasma carotenoid values during the initial depletion period. The liver stores of carotenoids were extremely low, which indicated a rapid decrease in this nutrient when no dietary source was available. The logarithms of the liver stores of carotenoids were found, as expected, to be linearly related to the logarithm of carotene intake and to be suppressed in a linear manner by the logarithm of vitamin A intake.

Liver stores of vitamin A, expressed as their logarithms were significantly influenced by initial stores of this nutrient as estimated by the average plasma vitamin A values during the initial depletion period. Both the unadjusted and adjusted values are presented in Table 3. These values were found to be linearly related to the logarithm of the intakes of both carotene and vitamin A plus ten. This relationship was best described by the formula:

$$\hat{y}_L = -0.663 + 0.720x_1 + 1.358x_2 + 0.020x_3$$

where $\hat{y}_L$ is the logarithm of the predicted liver stores of vitamin A in micrograms per 100 g. of liver and x_1 is the logarithm of the intake of carotene and x_2 the logarithm of the intake of vitamin A plus ten, both x_1 and x_2 in micrograms per pound of live weight per day, and x_3 is the average plasma vitamin A during the initial depletion period in micrograms per 100 ml. of plasma. It should be remembered that the values were obtained at the completion of the final, 4-week depletion period.

Depletion time and spinal fluid pressures. Depletion time was calculated for each calf as described by Teichman *et al. (22)*. This calculation utilized plasma vitamin A values from the 154th to the 175th day of age, the final depletion period, during which period the animals received the unsupplemented vitamin A depletion ration. In confirmation of Teichman *et al.*, the plasma vitamin A values were found to decrease in a linear manner with time. This function was best described by the formula:

$$\hat{y}_p = 22.71 - 2.0596t$$

where $\hat{y}_p$ equals the predicted plasma vitamin A values in micrograms per 100 ml. of plasma and t equals time in weeks. The regression coefficient, -2.0596, agrees very well with a similar value, -2.03, for 105-day-old male Holstein calves reported by Teichman *et al. (22)*. The coefficient was found to be independent of previous carotene and vitamin A levels of feeding and agreed with the previous report *(22)*.

Depletion time was calculated *(22)* for each calf, by using the formula

$$\hat{x}_D = 2.5 + \frac{(4 - Y)}{-2.0596}$$

where $\hat{x}_D$ equals the predicted depletion time in weeks, 2.5 equals the average number of weeks fed the depletion ration, 4 equals the depleted plasma vitamin A level desired for each calf expressed in micrograms per 100 ml. of plasma, Y equals the average plasma vitamin A value for each calf during its 4-week depletion period, and -2.0596 equals the linear rate of decrease in blood plasma vitamin A expressed in micrograms per 100 ml. plasma per week fed the depletion ration. These data are presented in Table 3. As would be expected, owing to the dependency of its calculation on the plasma vitamin A value, depletion time was similar to that criterion, in that it was found to be significantly related to initial stores of vitamin A as estimated by the average plasma vitamin A value during the initial depletion period. Depletion time was found to be linearly related to both carotene and vitamin A intake and was best described by the formula:

$$\hat{z}_D = 3.69 + 0.01x_1 + 0.12x_2 + 0.07x_3$$

where $\hat{z}_D$ is the predicted depletion time in weeks, x_1 and x_2 are the daily carotene and vitamin A intakes, respectively, expressed in micrograms per pound of live weight per day and x_3 is the average plasma vitamin A value during the initial depletion period, 35-63 days of age, in micrograms per 100 ml. of plasma.

Spinal fluid pressures, taken at the end of the final 4-week depletion period, were analyzed in terms of their logarithms. These data are presented in Table 3. Spinal fluid pressures tended to decrease with increases in either source of the vitamin. In view of the report by Moore *et al.*, *(13)*, that spinal fluid pressures in excess of 120 mm. of water were indicative of a vitamin A deficiency, the data collected in this experiment would indicate that the animals receiving 4 γ of vitamin A plus 20 γ of carotene and those which received 4 γ of vitamin A plus 50 γ of carotene per pound of live weight daily were deficient in vitamin A after 4 weeks of receiving a vitamin A depletion ration. It would thus appear that higher intakes of vitamin A and/or carotene would be desirable in practice, especially where periods of low vitamin A intake may be anticipated.

The practical significance of the results of this study is the finding that both sources of the vitamin may be included simultaneously in a ration without adverse effect on the utilization of either the provitamin, carotene, or vitamin A, per se. This is based on the additive response of plasma and liver vitamin A, as well as body stores of vitamin A (as estimated by predicted depletion time), to the simultaneous feeding of both sources of the nutrient. It is recognized that the inclusion of vitamin A, per se, resulted in a suppression of both plasma carotenoids and liver carotenoids, but the questionable biological values of body stores of the provitamin *(12)* make it impossible to evaluate the practical significance of this observation.

SUMMARY

Forty-eight, 63-day old, male Holstein calves, which had been partially depleted of their vitamin A stores, were fed a vitamin A depletion ration supplemented with one of four levels of carotene from artificially dehydrated alfalfa; 20, 50, 125, or 312.5 γ per pound of live weight daily plus one of four levels of vitamin A from a dry carrier: 4, 10, 25, or 62.5 γ per pound of live weight daily for a 12-week period. At the end of this period the animals received the unsupplemented depletion ration for 4 weeks so that body stores of vitamin A might be estimated. All animals were then slaughtered.

Analysis of the data indicated the following:

Growth as measured by increases in live weight, height at withers, heart girth, and girth of paunch was essentially unrelated to carotene and/or vitamin A intake.

The logarithms of plasma vitamin A increased in a linear manner with increases in the log-log of carotene and/or log-log vitamin A intake. Increases in the logarithms of carotene and vitamin A intake resulted in linear increases in the logarithms of liver vitamin A concentrations. Body stores of vitamin A as estimated by predicted depletion time expressed in weeks to deplete to blood plasma vitamin A levels of 4.0 γ per 100 ml., after carotene and vitamin A feeding, increased in a linear manner with increases in actual carotene and vitamin A intake. With respect to these criteria, the responses to carotene and vitamin A intake were additive and thus the simultaneous feeding of both sources of the vitamin was not found to affect adversely the utilization of either the provitamin or the vitamin itself. Quantitative expressions are presented.

The logarithms of plasma carotenoids were suppressed in a linear manner by increases in the log-log of vitamin A intake and increased in an essentially linear manner with increases in the log-log of carotene intake. A similar relationship was found to exist between the logarithm of liver carotenoids and the logarithms of the intakes of carotene and vitamin A.

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EFFECT OF SOME ANTIOXIDANTS ON THE UTILIZATION OF CAROTENE BY HOLSTEIN CALVES[1]

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Antioxidants have proven useful in decreasing losses of carotene in alfalfa during storage and have been reported to increase the apparent utilization of carotene by several animal species. The study herein reported was made to determine the effect of three antioxidants fed at the rate of 0.01% of the ration on the utilization of carotene by Holstein calves. *Editor.*

To insure adequate levels of vitamin A in the rations, artificially dehydrated alfalfa is frequently added because of the relatively high carotene (provitamin A) content. Currently, two problems exist in the efficient utilization of carotene from artificially dehydrated alfalfa, namely, the progressive destruction of carotene occurring during storage of the alfalfa and the relatively inefficient utilization of carotene by dairy cattle. The former can be prevented by a variety of means, such as storage at low temperatures or in an inert atmosphere and/or by the addition of certain antioxidants *(3)*. In contrast, little concrete information exists concerning possible means of increasing the utilization of carotene from alfalfa by dairy cattle.

N,N′-diphenyl-para-phenylenediamine, effective in preservation of carotene in alfalfa, has been reported to increase apparent carotene utilization in dairy cows *(26, 27)*. Two other antioxidants, 6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline and 2,5-ditertiarybutylhydroquinone, effective in the preservation of caro-

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tene in alfalfa have been found of relatively low toxicity to dairy calves *(9)*. Consumption of greater than 0.68 g. of antioxidant per pound dry matter intake was necessary before adverse effects were observed.

In animals other than dairy cattle, N,N′-diphenyl-para-phenylenediamine has been reported by three different groups of workers *(15, 17, 29)* to increase carotenoid pigmentation of the chick as well as carotene utilization from alfalfa by the chick. A fourth group of workers *(1)* reported no appreciable effect of this antioxidant on carotene utilization by the chick. In one of the aforementioned studies *(15)*, 2,6-ditertiarybutyl-4-methylphenol was found less effective in increasing carotene utilization than N,N′-diphenyl-para-phenylenediamine. Studies with the laboratory rat, as recently reviewed by High and coworkers *(10, 11)*, indicated that although a low dietary intake of an effective antioxidant increased carotene utilization, high dietary intakes of the same antioxidant decreased utilization by possible inhibition of the oxidative processes involved in the conversion of carotene to vitamin A.

Since antioxidants have proven useful in decreasing losses of carotene in alfalfa under storage and have been reported to increase the apparent utilization of carotene by several different animal species, the present study was undertaken to determine the effect of some antioxidants on utilization of carotene by Holstein calves.

EXPERIMENTAL PROCEDURES

Animals and rations. Thirty-two 1-day-old male Holstein calves, obtained from various Connecticut state institution herds during the period from December 1954 to March 1955, were placed on experiment and raised to 63 days of age according to standard procedures adopted at this station *(23, 24)*.

Beginning the 64th day of age, each calf was fed an intake of a vitamin A depletion ration to give an anticipated 7-day increase in live weight of 10 lb. *(23)*. This ration allowance was maintained for each calf until it was slaughtered. When the blood plasma vitamin A level for each calf decreased to or less than 10 γ/100 ml., the depletion ration was supplemented with one of two levels of carotene, 60 or 540 γ per pound live weight per day, from artificially dehydrated alfalfa leaf meal without or with the addition of one of three antioxidants, N,N′-diphenyl-para-phenylenediamine (DPPD), 6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline (Santoquin) or 2,5-ditertiarybutylhydroquinone (DTBH), fed at the rate of 0.01% of the ration. The levels of carotene were chosen to give a sufficiently wide range to cover intakes which might be met under most feeding practices, but sufficiently narrow that the expected responses of the carotene and vitamin A criteria would be within the linear range. The particular antioxidants used were chosen on the basis of their effectiveness in decreasing losses of carotene in alfalfa under storage *(16, 28)* and their availability to the feed industry. The level of antioxidant feeding was considerably lower than that used for preservation of carotene in alfalfa under storage, 0.1 to 0.2% *(16, 28)*, but found to be effective in the poultry studies *(15, 17, 29)* and approximately equivalent to

such levels when alfalfa was added to the ration at rates of 5 to 10%. The first eight calves to arrive at the research barn, Replicate I, were assigned to one of the eight treatments according to random allotment. This procedure was repeated until four replicates of eight calves each had been assigned to their respective treatments. The average age at the beginning of the supplement feeding period was 79.8 ± 1.3 days. The duration of this period, the comparison period, was 12 successive 7-day intervals for each calf.

Upon completion of the comparison period, the carotene supplement was removed from the rations of all calves and the antioxidants from the rations of half of the calves, Replicates I and III. This regime, the final-depletion period, was maintained until each calf's blood plasma vitamin A concentration decreased to less than 4.0 γ /100 ml. for two consecutive 7-day intervals *(12)*. This was for the purpose of observing vitamin A depletion time.

The calculation of the amount of artificially dehydrated alfalfa leaf meal fed each calf, its weighing, and the method of incorporating it into the depletion ration allowance were as previously described *(23)*. The antioxidants were incorporated by thorough mixing into a finely ground depletion ration mixture such that the concentration was 45.4 mg. per gram of mixture. Premixes were weighed and incorporated into the depletion ration allowance as was the artificially dehydrated alfalfa leaf meal.

Scours, when accompanied by rectal temperatures of 103° F. or above, were treated with an initial intravenous injection of 25% solution of sulfamethazine at the rate of 26.3 ml. per 100 lb. live weight, followed by oral administration of sulfamethazine oblets at the rate of 6.5 g. per 100 lb. live weight for two additional days.

Average minimum and maximum barn temperatures with their standard deviations were respectively: 59.9 ± 8.9 and 72.2 ± 9.2° F. for the comparison period and 60.3 ± 8.6 and 72.2 ± 8.9° F. for the final depletion period.

Observations and analyses. All feeds fed and refused were weighed to the nearest 0.1 lb. Live weights were recorded to the nearest pound at successive 7-day intervals throughout the entire experiment, and height at withers, heart girth, and circumference of barrel to the nearest ¼ in. were obtained at the start and completion of the comparison period. Venous blood samples for plasma carotenoid and vitamin A determinations *(14)* were obtained on the fourth and seventh days of each calf's 7-day intervals during the initial vitamin A depletion period and thereafter at successive 7-day intervals. Spinal fluid pressures *(18)* were obtained at the completion of the comparison period and thereafter at 14-day intervals.

The day prior to slaughter, a venous blood sample was obtained for plasma tocopherol determination *(20, 21)*. Immediately after slaughter, the liver was removed for carotenoid and vitamin A *(7)*, lipid *(22)*, and total tocopherol *(13)* determinations.

Samples of the feeds were obtained at successive 4-week intervals during the course of the experiment. Samples of the artificially dehydrated alfalfa

leaf meal were taken at weekly intervals during all calves' comparison periods. All samples were analyzed for proximate constituents by A.O.A.C. procedures *(2)* and the alfalfa leaf meal for carotene by the official A.O.A.C. procedures *(2)* except that extraction of the pigments employed the alternative extraction procedure of Method II, first action, in lieu of hot extraction. The alfalfa leaf meal contained 0.239 ± 0.003 mg. of carotene per gram. Average chemical composition of the feeds fed are contained in Table 1.

Standard procedures *(8, 25)* were employed in the statistical analyses of the data.

TABLE 1
Average chemical composition of feeds

	Per cent dry matter	Per cent of dry matter					
		Crude protein	Ether extract	Crude fiber	N.F.E.	Ash	Carotene (*mg/lb.*)
Alfalfa hay chopped	92.34 ± 0.35[a]	19.24 ± 0.49	1.60 ± 0.13	30.28 ± 0.70	41.16 ± 0.81	7.60 ± 0.32	7.11 ± 0.32
Calf starter	92.20 ± 0.40	23.13 ± 0.12	5.46 ± 0.17	6.79 ± 0.08	56.28 ± 0.27	8.34 ± 0.08	2.54 ± 0.07
Vitamin A depletion mixture	90.34 ± 0.83	15.60 ± 0.25	2.04 ± 0.15	13.66 ± 0.67	61.98 ± 0.49	6.71 ± 0.15	0.09 ± 0.02
Artificially-dehydrated alfalfa leaf meal	91.75 ± 1.10	24.18 ± 0.63	2.95 ± 0.15	20.96 ± 0.35	38.48 ± 0.54	13.38 ± 0.32	108.32 ± 1.29

[a] Standard error of the mean.

RESULTS

Feed consumption, growth, and health. Feed refusals, days with scours, and the number of treatments with sulfamethazine for scours were relatively few and unaffected by any of the treatments either during the comparison or final depletion periods.

Average initial live weight, height at withers, heart girth, and circumference of barrel with their standard deviations per calf were, respectively, at the start of the comparison period: 202 ± 20 lb., 34.6 ± 1.0 in., 38.2 ± 1.3 in., and 47.6 ± 1.7 in. Increases during the 12-week comparison period of these criteria, which were found not to be related to treatment, were 154 ± 12 lb., 5.2 ± 0.9 in., 8.2 ± 1.2 in., and 9.9 ± 2.3 in., respectively. During the final depletion period, the mean increase in live weight was 14.75 ± 0.24 lb. per 7-day period. As for the comparison period, increases in live weight were found not to be closely associated with carotene level or antioxidants.

The average spinal fluid pressure taken during the last 7-day interval of the comparison period was 93.1 ± 8.1 mm. H_2O. The geometric mean derived from values taken within the 14-day age intervals previous to the decrease of each calf's blood plasma vitamin A concentration to 4.0 γ/100 ml. during the final

depletion period, was 159 mm. H_2O [geometric mean transformed from average log (mm. spinal fluid pressure), 2.201 ± 0.117]. With one exception, this criterion was found to be unaffected by treatment. The one exception occurred in the calves fed DPPD, and these had on the average significantly lower values during the final depletion period than the controls or the other two antioxidants [controls had a mean log (spinal fluid pressure) of 2.220, DPPD 2.072, Santoquin 2.237, and DTBH 2.273 with the least significant difference at P = 0.05 of 0.124].

TABLE 2

Effect of some antioxidants on concentration of plasma carotenoids and vitamin A during carotene feeding

	Plasma carotenoids			Plasma vitamin A		
	Initial[a]	Comparison period[b]		Initial[a]	Comparison period[b]	
		Actual	Log		Actual	Log
	(γ/100 ml. plasma)					
60 γ carotene intake						
No antioxidant[c]	9	27	1.561	9.2	19.0	1.272
DPPD	8	33	1.607	8.8	16.9	1.215
Santoquin	6	34	1.618	7.9	20.8	1.307
DTBH	13	35	1.641	9.2	17.1	1.228
540 γ carotene intake						
No antioxidant	12	90	1.964	8.5	27.7	1.434
DPPD	13	134	2.140	7.2	23.1	1.356
Santoquin	12	147	2.171	9.8	27.8	1.440
DTBH	12	138	2.145	8.4	27.5	1.428
Standard deviation of a calf	8	.....	0.109	1.4	.....	0.062

[a] Value for the day prior to supplementation with carotene and antioxidants.
[b] Average value for 12-wk. comparison period during which carotene and antioxidants were fed.
[c] Four calves per treatment group.

Carotenoids, vitamin A, and tocopherol. In Table 2 are presented plasma carotenoid and plasma vitamin A values for the comparison period; in Table 3, rates of change with time of the same criteria during the final depletion period, as well as vitamin A depletion time. Table 4 contains data on liver weight, liver lipids and liver carotenoids, vitamin A, total tocopherol, and plasma tocopherol, these criteria being observed at the completion of the final depletion period.

All antioxidants (Table 2) resulted in significantly greater concentrations of plasma carotenoids during the comparison period than those observed in the control calves. Only slight differences were found among treatment means during the same period for concentrations of vitamin A in the plasma.

During the final depletion period, the rates of decrease of plasma carotenoids with time (Table 3) were slightly greater in the calves fed antioxidants than in the control calves (as evidenced by the greater linear regression coefficients). The inclusion of the antioxidants in the ration (+ calves) during this final depletion period retarded to some extent the decrease in plasma carotenoids. Inappreciable differences in the rates of change of plasma vitamin A concentrations were noted among treatments for calves fed previously 60 γ carotene. In

TABLE 3

Effect of some antioxidants on time trends of plasma carotenoids and vitamin A during vitamin A depletion and vitamin A depletion time

	Antioxidant fed during depletion period	No. of calves	Plasma carotenoids		Plasma vitamin A	Vitamin A depletion time
			Linear trend[a]	Quadratic trend[a]	Linear trend[a]	
			(γ/100 ml/7 d. period)			*(wk.)*
60 γ carotene intake						
No antioxidant	−	4	− 2.125 ± 1.022[b]	0.469 ± 0.346[b]	− 1.796 ± 0.478[b]	4.6 ± 1.0[c]
DPPD	−	2	− 5.782 ± 1.743	1.253 ± 0.877	− 2.084 ± 0.815	4.5 ± 1.5
	+	2	− 5.000 ± 2.890	1.500 ± 2.221	− 1.970 ± 1.351	4.2 ± 1.5
Santoquin	−	2	− 6.500 ± 2.890	2.750 ± 2.221	− 2.480 ± 1.351	3.4 ± 1.5
	+	2	− 3.600 ± 1.743	0.897 ± 0.877	− 2.278 ± 0.815	4.8 ± 1.5
DTBH	−	2	− 4.044 ± 1.927	0.768 ± 0.977	− 1.982 ± 0.901	4.4 ± 1.5
	+	2	− 5.100 ± 2.043	1.571 ± 1.187	− 1.945 ± 0.955	4.6 ± 1.5
540 γ carotene intake						
No antioxidant	−	4	− 4.676 ± 0.444	1.098 ± 0.105	− 1.921 ± 0.207	10.2 ± 1.0
DPPD	−	2	− 4.295 ± 0.463	0.975 ± 0.089	− 1.427 ± 0.216	12.6 ± 1.5
	+	2	− 4.912 ± 0.507	0.999 ± 0.109	− 1.139 ± 0.237	12.1 ± 1.5
Santoquin	−	2	− 6.733 ± 0.535	1.236 ± 0.117	− 1.773 ± 0.250	11.5 ± 1.5
	+	2	− 6.222 ± 0.509	1.063 ± 0.096	− 1.551 ± 0.238	11.3 ± 1.5
DTBH	−	2	− 5.468 ± 0.416	0.873 ± 0.075	− 1.720 ± 0.194	13.8 ± 1.5
	+	2	− 4.247 ± 0.575	0.838 ± 0.134	− 1.239 ± 0.269	11.0 ± 1.5
60 + 540 γ carotene intake						
No antioxidant	−	8	− 4.271 ± 0.407	1.046 ± 0.100	− 1.901 ± 0.190	7.4 ± 0.7
DPPD	−	4	− 4.393 ± 0.447	0.978 ± 0.089	− 1.470 ± 0.209	8.6 ± 1.0
	+	4	− 4.915 ± 0.499	1.000 ± 0.109	− 1.164 ± 0.233	8.2 ± 1.0
Santoquin	−	4	− 6.725 ± 0.526	1.240 ± 0.117	− 1.796 ± 0.246	7.4 ± 1.0
	+	4	− 6.016 ± 0.489	1.061 ± 0.095	− 1.608 ± 0.220	8.1 ± 1.0
DTBH	−	4	− 5.404 ± 0.406	0.873 ± 0.075	− 1.731 ± 0.190	9.1 ± 1.0
	+	4	− 4.310 ± 0.553	0.847 ± 0.133	− 1.291 ± 0.259	7.8 ± 1.0

[a] Expressed as orthogonal polynomial coefficients *(25)*.
[b] Slope ± its standard error.
[c] Mean ± its standard error.

the calves fed previously 540 γ carotene, antioxidants reduced the rate of decrease of plasma vitamin A. The inclusion of antioxidants in the ration during this depletion period consistently retarded decreases of plasma vitamin A with time, and the decreases due to the antioxidants were of greater magnitude in the calves fed previously 540 γ carotene than in those fed 60 γ.

Vitamin A depletion time (Table 3) was inappreciably affected by antioxidants in calves fed previously 60 γ carotene but was increased by antioxidants in calves fed 540 γ carotene. The inclusion of antioxidants in the ration during the final depletion period had inappreciable effect on the vitamin A depletion time.

The weights of the livers of calves fed previously 540 γ carotene (Table 4) were greater at the completion of the vitamin A depletion period than were those for calves fed 60 γ carotene. This was largely due to the longer depletion period of the former group, which resulted in greater live weight and thus greater liver weight. The concentrations of lipids in the livers of calves fed antioxidants either only during the comparison period or during both the comparison and final depletion periods appeared to be slightly greater than those in the control calves. Carotenoids, vitamin A, and total tocopherol concentrations were greater in the livers of calves fed previously 540 γ carotene than in calves fed 60 γ. Antioxidants fed only during the comparison period or during the comparison and final depletion periods resulted in slightly greater concentrations of these substances in the liver. Inappreciable differences were observed for plasma tocopherols.

DISCUSSION

The data of this study indicated that the three antioxidants, N,N′-diphenyl-para-phenylenediamine (DPPD), 6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline (Santoquin) and 2,5-ditertiarybutylhydroquinone (DTBH), resulted in increases in the utilization of carotene from alfalfa. The main location of activity of these antioxidants with respect to the utilization of carotene appeared to be the digestive tract of the calves, with limited evidence of activity within the body proper. This assumption was based on the small differences in response criteria between calves fed the antioxidants during only the comparison period when carotene from alfalfa was fed and calves fed the antioxidants during both the comparison and final depletion periods. These data with respect to increased utilization of carotene are therefore in agreement with the previous reports for dairy cattle *(26, 27)* and other species *(15, 19, 29)*.

The possible slight sparing action of these antioxidants on total tocopherol concentration in the liver and on increased lipid concentrations of the liver are in agreement with a previous report on the effect of DPPD in poultry *(6)* and on the effect of DPPD on the fat percentage of milk of lactating cows *(26, 27)*. Thus the data contained herein gave added support to the possible action of antioxidants on fat metabolism.

TABLE 4

Effect of some antioxidants on liver lipids, carotenoids, vitamin A and tocopherol, and plasma tocopherol at the completion of a vitamin A depletion period[a]

	Antioxidant fed during depletion period	No. of calves	Liver (wet basis)								Plasma tocopherols
			Weight	Lipids	Carotenoids		Vitamin A		Total tocopherols		
					Actual	Log	Actual	Log	Actual	Log[b]	
			(g.)	*(g /100 g.)*	*(γ/100 g.)*				*(mg /100 g.)*		*(mg /100 ml.)*
60 γ carotene intake											
No antioxidant	−	4	3114	3.78	14	1.138	7	0.803	0.24	1.369	0.160
DPPD	−	2	3416	3.96	18	1.254	2	0.392	0.32	1.497	0.234
	+	2	2908	4.16	18	1.242	8	0.878	0.20	1.307	0.161
Santoquin	−	2	3220	3.74	18	1.184	4	0.548	0.23	1.345	0.129
	+	2	3672	4.06	12	1.057	4	0.574	0.25	1.374	0.150
DTBH	−	2	3235	3.86	16	1.190	7	0.836	0.23	1.333	0.184
	+	2	3287	3.75	18	1.238	6	0.758	0.31	1.483	0.209
540 γ carotene intake											
No antioxidant	−	4	3868	3.86	19	1.277	6	0.696	0.22	1.286	0.178
DPPD	−	2	3936	3.85	14	1.130	23	1.314	0.40	1.604	0.200
	+	2	4242	3.84	27	1.428	26	1.344	0.18	1.221	0.158
Santoquin	−	2	3818	3.79	20	1.301	12	1.056	0.40	1.572	0.158
	+	2	4030	4.20	20	1.310	10	0.946	0.28	1.434	0.183
DTBH	−	2	4156	4.17	22	1.340	12	1.037	0.38	1.556	0.159
	+	2	3684	4.20	22	1.332	15	1.123	0.39	1.580	0.173
Standard deviation of a calf			296	0.28	....	0.097	....	0.244	....	0.147	0.046

[a] All values, two 7-day intervals after blood plasma vitamin A concentration had decreased for each calf to less than 4.0 γ /100 ml.

[b] Log [100 (mg. tocopherol/100 g. liver)].

The inappreciable response to antioxidants of calves fed the 60 γ carotene intake in contrast to the relatively greater response of those fed the 540 γ carotene intake was of interest. Two explanations appeared possible: *(a)* that the precision of measurement of the various criteria was not sufficiently great and the number of replications (calves) was not sufficiently large to detect differences or *(b)* since efficiency of utilization of carotene is already high at the lower intake, inappreciable response to antioxidants might be expected. The first explanation appeared more plausible.

Since it has been established that Santoquin and DPPD are present in the milk of cows fed these antioxidants *(3, 27)* and DPPD is present in the eggs of hens fed this antioxidant *(19)*, it was reasonable to assume that the antioxidants studied here are present in the tissues of the calves. The possible interference of these substances in the estimation of carotenoids and vitamin A was therefore explored for reasons previously reviewed *(27)*. Absorbancy at 440 mμ for the estimation of carotenoids as determined with an Evelyn colorimeter was increased 0.00003 ± 0.00002 per microgram of feed grade DPPD, 0.00105 ± 0.00030 per microgram of feed grade Santoquin, and 0.00049 ± 0.00028 per microgram of feed grade DTBH. Similar values at 620 mμ for the estimation of vitamin A by the Carr-Price reaction were, respectively, 0.00032 ± 0.00003, 0.00061 ± 0.00003, and 0.00014 ± 0.00035. Thus the possibility existed for some overestimation of carotenoid and vitamin A values.

The possible overestimation of plasma vitamin A in calves not receiving the antioxidants during the final depletion period would appear to be inappreciable. This is based on the observation *(26)* that only a 2-week period was necessary after the withdrawal of DPPD from lactating cows' ration, before the effectiveness in preventing the copper induced oxidized milk flavor defect was no longer apparent and presumably DPPD was no longer present in the body tissues. Therefore vitamin A depletion times of 4 weeks in calves fed previously 60 γ carotene and of 10 weeks in those fed previously 540 γ carotene would have allowed sufficient time for the antioxidants fed to have been eliminated from the body. Possible interference of the antioxidants studied on the tocopherol determinations was not investigated. However, it is known that DPPD is strongly held on the chromatographic absorbent, Florex XXS, and is not eluted by ethyl alcohol or strong acids *(5)*. Therefore in calves fed DPPD, interference in estimating the total tocopherol concentration of the livers was not likely.

SUMMARY

The effect of three antioxidants, N,N′-diphenyl-para-phenylenediamine (DPPD), 6-ethoxy-2,2-4-trimethyl-1,2-dihydroquinoline (Santoquin), and 2,5-ditertiarybutylhydroquinone (DTBH), on the utilization of carotene from alfalfa was studied with 32 male Holstein calves. These calves were raised to 63 days of age on a standard limited whole milk, limited starter, and free-choice alfalfa hay regime. On the 64th day of age each calf was fed an intake of a vitamin A depletion ration to provide an anticipated rate of gain in live weight of 10 lb.

per 7-day period. When the blood plasma vitamin A level for each calf had decreased to or less than 10.0 γ/100 ml., each calf was then fed either 60 or 540 γ carotene from artificially dehydrated alfalfa per pound live weight per day without or with the addition of one of the three antioxidants at 0.01% of the ration for 12 consecutive 7-day intervals. Upon completion of this period, the comparison period, the carotene was removed from the rations of all calves and the antioxidants from the rations of half the calves previously receiving them. This regime, the final depletion period, was maintained until each calf's blood plasma vitamin A concentration decreased to less than 4.0 γ per 100 ml. for two consecutive 7-day intervals, at which time the calf was slaughtered. The following results were found:

1. Calves fed antioxidants during the comparison period, when carotene from alfalfa was included in the ration, had higher concentrations of carotenoids in the plasma than did the control calves.

2. Calves fed antioxidants during only the comparison period and calves fed antioxidants during both the comparison and final depletion periods had longer vitamin A depletion times than the control calves. This finding indicated greater vitamin A storage at the end of the carotene feeding period and thus greater utilization of carotene.

3. Based on the inappreciable differences of the responses between calves fed antioxidants during only the comparison period and those fed antioxidants during both the comparison and final depletion periods, it was suggested that the main site of activity of the antioxidants with respect to their effect on utilization of carotene was the gut.

4. Upon the completion of the final depletion period, liver lipids and liver total tocopherol concentrations were slightly greater in the calves fed antioxidants.

5. At the end of the final depletion period calves fed DPPD had significantly lower spinal fluid pressures than the controls or those fed Santoquin or DTBH.

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Artificially dehydrated alfalfa leaf meal was obtained from the National Alfalfa Dehydrating and Milling Co., Kansas City, Mo.; it contained no additives, such as antioxidants or oils. DPPD, feed grade, was obtained from the B. F. Goodrich Chemical Co., Cleveland, Ohio, through the courtesy of R. E. Althaus. Santoquin, Lot V-1195, was obtained from the Monsanto Chemical Co., through the courtesy of J. S. Ruhoff. DTBH, feed grade, was obtained from the Eastman Chemical Products, Inc., Kingsport, Tenn., through the courtesy of W. M. Gearheart. Sulfamethazine, American Cyanamid Co. "Sulmet," was kindly furnished through the courtesy of R. F. Elliott.

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PROTEIN PRODUCTION IN THE BOVINE. BREED AND INDIVIDUAL VARIATIONS IN THE SPECIFIC PROTEIN CONSTITUENTS OF MILK[1]

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For biological as well as technological reasons a greater knowledge of the relation of breed of cattle to the production of the major proteins of milk is important. The milk from five to seven cows from each of five major breeds was used for electrophoretic analysis. The amounts of α-, β- and γ-casein, α-lactalbumin, β-lactoglobulin, the immune globulins, and serum albumin present in each milk were determined. *Editor.*

The various breeds of dairy cattle produce significantly different amounts of protein in their milk, and individuals within the breeds show considerable variation *(9, 10, 11, 12, 14)*. In general the breeds producing less total milk but having a higher fat content also produce milk containing more protein. Studies on the proteins of skimmilk have been confined to an evaluation of the variations in the total protein or of the total casein and serum (whey) protein fractions. In some studies the serum proteins have been further separated into "lactoglobulin," "lactalbumin," and "proteose-peptone" fractions on the basis of their heat stability and solubility in concentrated salt solutions *(10, 11, 12, 14)*. These fractionation procedures and terminology have become outmoded by the recent isolations in purified or crystalline form of the major specific protein constituents of milk *(3, 8, 13)*.

The evaluation of the specific milk proteins may be accomplished most easily by an electrophoretic analysis of the isolated casein and the isolated serum proteins even though the number of samples that can be analyzed by this procedure is limited. Casein on electrophoresis at pH 8.6 separates into three components (α-, β-, and γ-casein) *(8)*. On electrophoresis at pH 8.6 the serum proteins separate into eight or more fractions; however, four of these, the immune globulins, α-lactalbumin, β-lactoglobulin, and blood serum albumin, compose up to 85% of the normal milk serum protein *(3, 8)*. The immune globulins (euglobulin and pseudoglobulin) compose most of the classical "lactoglobulin" fraction and are probably identical to some of the immune globulins of blood *(1, 5, 8, 13)*. α-Lactalbumin, β-lactoglobulin, and serum albumin have been isolated in crystalline form from milk and compose essentially the classical "lactalbumin" fraction *(3, 8)*. The serum albumin of milk is identical with the

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serum albumin of blood *(2)*. In addition there are enzymes, unidentified minor proteins, and unspecific "proteose-peptone" materials present *(3, 8, 12)*.

The biological and technological importance of each of the major specific proteins prompted a study to determine if there are differences between the various breeds of dairy cattle in their production of these proteins. The amounts of α-, β-, and γ-casein, α-lactalbumin, β-lactoglobulin, the immune globulins, and serum albumin in milk were determined in this study.

METHODS AND PROCEDURES

Studies in this laboratory have indicated that the stage of lactation has an effect on the total protein production per day and the ratio of the amounts of the specific proteins in the milk relative to each other *(4, 6)*. The relative amounts of these proteins in the milk vary greatly at the start and near the end of the lactation period; thus the milk samples for this study were taken from cows that had been in lactation 2 to 6 months.

Samples were taken from 28 healthy cows in the University herd including five to seven cows from each of the five major dairy breeds. Two from each breed were in their first lactations. The samples of milk were collected at five weekly intervals during July and August from groups of five to seven cows, including at least one cow from each breed. Samples of milk were taken from each cow at the morning and evening milkings of two consecutive days; the four samples were composited proportional to the weight of each milking. The composited sample of milk was centrifuged for 20 minutes at 2000 × gravity in 300-ml. polyethylene bottles and the skimmilk was pipetted from beneath the layer of fat. An aliquot of 200 ml. of skimmilk was used for the preparation of the serum proteins for the electrophoretic analysis according to previously described procedures *(3)*.

Casein was prepared for the electrophoretic analysis by precipitating it from 25 ml. of skimmilk with 10% acetic acid at pH 4.65, redispersing in sodium-potassium phosphate buffer (pH 6.9, ionic strength of 0.1), reprecipitating with 10% acetic acid, and redispersing in sodium veronal buffer (pH 8.6, ionic strength of 0.1) to give a concentration of 2 to 4%. Centrifugation in 50-ml. plastic centrifuge tubes and homogenization in a glass tissue grinder were used for the precipitation and redispersion steps, respectively. The casein solutions were centrifuged at 2000 × gravity for 30 minutes to remove traces of fat and other opaque material. An aliquot of 17.6 ml. was dialyzed against 250 ml. of veronal buffer (pH 8.6, ionic strength of 0.1) for 12 to 24 hours at 4° C. and then against 500 ml. of the same strength buffer for an additional 24-hour period. At the time of the electrophoretic analysis the casein solutions were diluted with equilibrated buffer to the desired concentration. The concentration of the solution actually used in the electrophoretic analysis was determined by a semi-micro Kjeldahl analysis.

The electrophoretic analyses were all conducted in sodium veronal buffer (pH 8.6, ionic strength of 0.1, 0.12 *M*) for 5,580 seconds at 1.2° C. with a field

strength of 7.8 volts per square centimeter as previously described *(3, 7)*. The casein solutions were analyzed at a concentration of about 1.5% and the serum protein solutions at 2-3% protein. Difficulties encountered with the isolation procedure and the electrophoretic analyses resulted in the loss of some samples. In some instances, only the casein or the serum protein fraction for a given cow was carried through the complete analysis without loss.

The electrophoretic patterns of the serum proteins were divided into four areas representing the immune globulins, α-lactalbumin, β-lactoglobulin, and serum albumin *(3)*. Since in addition to the usual electrophoretic anomalies other minor protein components are present, the actual amounts of each constituent are probably 5 to 15% lower than the figures reported *(3)*.

TABLE 1

Within and between breeds components of variance for the protein constituents of milk

Protein component	Components of variance	
	Within breed	Between breeds
	On a basis of grams per 100 ml. skimmilk	
Casein:		
α-casein	0.0831	0.0166
β-casein	0.0204	0.0091*
γ-casein	0.0017	0.0019**
Total casein	0.1563	0.0131
Serum proteins:		
Immune globulins	0.0044	trace[a]
α-lactalbumin	0.0008	trace[a]
β-lactoglobulin	0.0063	0.0001
Serum albumin	0.0001	trace[a]
Total serum proteins	0.0143	0.0004

* Derived from a mean square that was significant at the 0.05 level of probability.
** Derived from a mean square that was significant at the 0.01 level of probability.
[a] Components were positive but the first five decimal places were zeros.

RESULTS

The components of variance in Table 1 indicate that there was considerably more variation between cows within the same breed than between the breeds.

TABLE 2

Analysis of variance of β-casein

Source	Degrees of freedom	Mean squares	Expected mean squares
Total	24		
Between breeds	4	0.0654*	$(\sigma^2_w + 4.94\ \sigma^2_\beta)$[a]
1. Hol. vs (Ayr. + Guer. + Jer. + B. Sw.)	1	0.2413**	
2. Ayr. vs (Guer. + Jer. + B. Sw.)	1	0.0066	
3. B. Sw. vs (Guer. + Jer.)	1	0.0051	
4. Guer. vs Jer.	1	0.0091	
Within breeds	20	0.0204	(σ^2_w)

[a] For the between-breed mean squares involving the serum proteins the coefficient of β^2 was 5.17.

The components of variance were derived from analyses of variance tables similar to that shown in Table 2. For every protein constituent the four degrees of freedom between breeds were broken down into individual comparisons as is shown in Table 2, and the results of these comparisons are shown in Tables 3 and 4.

TABLE 3

The average amounts of proteins present in the skimmilk of the various breeds

Protein component	Ayrshire	Brown Swiss	Guernsey	Holstein	Jersey	Within breed standard deviations
	(g. per 100 ml.)					
Casein: (No. samples)	(5)	(4)	(5)	(7)	(4)	(25)
α-casein	1.71[b]	1.83	1.92	1.58[a]	1.83	0.29
β-casein	0.85	0.84	0.82	0.60[a]	0.76	0.14
γ-casein	0.08[b]	0.11	0.14	0.20[a]	0.13	0.04
Total	2.64	2.78	2.88	2.38[a]	2.72	0.39
Serum proteins: (No. samples)	(5)	(4)	(6)	(6)	(5)	(26)
Immune globulins	0.06	0.07	0.08	0.09	0.08	0.07
α-lactalbumin	0.11	0.11	0.11	0.13	0.15	0.03
β-lactoglobulin	0.31	0.31	0.35	0.30	0.39	0.08
Serum albumin	0.03	0.04	0.04	0.04	0.04	0.01
Total	0.51	0.53	0.58	0.56	0.66	0.12

[a] Comparison 1, Table 2, was significant for total casein and highly significant for α-, β-, and γ-casein.
[b] Comparison 2, Table 2, was highly significant for α- and γ-casein.

TABLE 4

The ratio of the specific proteins present in casein and the serum proteins isolated from the various breeds

Protein component	Ayrshire	Brown Swiss	Guernsey	Holstein	Jersey	Within breed standard deviations
			(% of total casein or serum proteins)			
Casein:						
α-casein	64.6	65.8	66.4	66.3	67.2	2.92
β-casein	32.2[b]	30.1	28.3	25.2[a]	27.9	3.04
γ-casein	3.1	4.1	5.2	8.5[a]	4.8	1.56
Serum proteins:						
Immune globulins	11.5	13.0	13.5	15.8	12.8	2.71
α-lactalbumin	21.7	21.3	19.7	23.3	22.2	2.18
β-lactoglobulin	61.3	58.9	59.8	54.4[a]	59.6	3.55
Serum albumin	5.5[b]	6.7	6.8	6.6	5.6	.65

[a] Comparison 1, Table 2, was significant for β- and γ-casein and for β-lactoglobulin.
[b] Comparison 2, Table 2, was significant for β-casein and for serum albumin.

A. Amounts of the proteins present in the milk. The breed averages shown in Table 3 indicate that the milk of the Holstein cows contained significantly less total casein, α-casein, and β-casein but significantly more γ-casein than the milk of the cows of the four other breeds. The Ayrshire milk contained significantly less α-casein than the milk of the breeds other than Holstein, and significantly less γ-casein than the milk of the four other breeds.

No significant differences were shown between the breeds in the total or in any of the specific serum proteins; however, the power of these tests of significance is low. It should be noted that the milk from the Holstein cows contained on the average less β-lactoglobulin and more immune globulins than the milk of the other four breeds.

B. Relative amounts of the specific proteins present in the casein and the serum proteins. Significant differences were found between the breeds in respect to the ratio of the specific proteins present in the casein and the serum proteins. The data in Table 4 show that the casein from Holstein cows contained significantly less β-casein but significantly more γ-casein than the casein from the other four breeds. The casein from the Ayrshire cows contained significantly more β-casein than the casein from the other four breeds. The α-casein content of the total casein was practically the same (64.6 to 67.2%) for all of the breeds. Practically all of the variation shown was in the relative amounts of β-casein and γ-casein.

The serum proteins from the Holstein cows contained significantly less β-lactoglobulin than did the serum protein of the other four breeds, and, though not significant, they contained a higher average content of immune globulins and α-lactalbumin. The serum proteins of the Ayrshire and Jersey breeds contained significantly less serum albumin than the serum proteins of the three other breeds.

DISCUSSION

Since it was apparent that the relative and actual amounts of the specific milk proteins of the individual animals varied, the within-breed correlation coefficients between the specific constituents were calculated from the data for all animals and are shown in Table 5. The highly significant correlation between the total casein and the total serum proteins indicates that cows that produced milk high in casein composition also produced milk high in total serum proteins. The high correlations between α-casein, β-casein, β-lactoglobulin and α-lactalbumin thus would be expected since these proteins compose most of the total casein and the total serum proteins. This suggests that the synthesis of these four major constituents in the mammary gland might be closely related. The amount of γ-casein or milk serum albumin present bears no significant relationship to any of the other constituents. This agrees with the observations *(6)* that the pattern of production of these constituents during the lactation period is more erratic and bears little relationship to the production of the other protein constituents. It is of interest to note that, although not significant, there is a negative correlation between the amount of γ-casein present and the amount of immune globulins, β-lactoglobulin, and milk serum albumin.

These studies indicate that the amounts of the specific proteins present in the milk of the Holstein breed appear to differ most from the other breeds, whereas the Brown Swiss, Guernsey, and Jersey breeds are remarkably alike. The α-casein content of the total casein was relatively constant for all of the individual cows. Other than the Holstein breed, which contained a significantly

TABLE 5

Within-breed correlations between the protein constituents of milk

Correlations between grams in 100 ml. of skimmilk	Correlation coefficient	Degrees of freedom
Total casein and total serum proteins	0.676**	17
α-casein and β-casein	0.599**	19
α-casein and γ-casein	0.191	19
α-casein and immune globulins	0.364	17
α-casein and α-lactalbumin	0.584**	17
α-casein and β-lactoglobulin	0.538*	17
α-casein and serum albumin	0.125	17
β-casein and γ-casein	0.067	19
β-casein and immune globulins	0.357	17
β-casein and α-lactalbumin	0.680**	17
β-casein and β-lactoglobulin	0.550*	17
β-casein and serum albumin	0.248	17
γ-casein and immune globulins	−0.259	17
γ-casein and α-lactalbumin	0.113	17
γ-casein and β-lactoglobulin	−0.243	17
γ-casein and serum albumin	−0.052	17
immune globulins and α-lactalbumin	0.487*	20
immune globulins and β-lactoglobulin	0.504*	20
immune globulins and serum albumin	0.489*	20
α-lactalbumin and β-lactoglobulin	0.845**	20
α-lactalbumin and serum albumin	0.310	20
β-lactoglobulin and serum albumin	0.327	20

* Significant at the 0.05 level of probability.
** Significant at the 0.01 level of probability.

lower percentage of β-lactoglobulin in the total serum proteins, the four other breed averages for β-lactoglobulin were remarkably close. It also has been noted that the β-lactoglobulin content of the serum proteins, although varying between cows, does not vary much for the individual cow in the middle ranges of the lactation period *(6)*.

SUMMARY

An evaluation of the specific milk proteins of the milk of 28 dairy cows including the five major breeds was made by a quantitative electrophoretic procedure. Large individual variations and also significant breed differences were found. The milk of Holstein cows contained significantly less total casein, α-casein and β-casein, but significantly more γ-casein than did the milk of the four other breeds. Ayrshire milk contained significantly less α-casein than did the milk of the Brown Swiss, Guernsey, and Jersey breeds and less γ-casein than did the milk of the breeds other than the Holstein.

All of the breeds produced casein containing approximately the same amount of α-casein; however, the casein of the Holstein breed contained significantly less β-casein and more γ-casein, whereas casein from the Ayrshire breed contained significantly more β-casein. The serum proteins of Holstein milk also contained significantly less β-lactoglobulin.

High correlations were found between the amounts in skimmilk of α-casein, β-casein, α-lactalbumin, β-lactoglobulin and to a lesser extent the immune globulins, but little relationship was found between the above constituents and the amount of γ-casein or serum albumin.

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DILUTERS FOR BOVINE SEMEN. IX. MOTILITY OF BOVINE SPERMATOZOA IN MILK-GLYCINE AND EGG YOLK–GLYCINE DILUENTS WITH AND WITHOUT GLYCEROL[1]

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The authors have studied the feasibility of using combinations of glycine and skimmilk as a diluent for bovine semen. Motility estimations over a 20-day storage period were made with a microscope equipped with a thermostage. The addition of glycerol or fructose to reconstituted NFDMS–glycine diluent gave improved results. *Editor.*

The motility and fertilizing capacity of sea urchin spermatozoa are beneficially affected by the inclusion of glycine in the diluting fluid *(10, 11)*. Although attempts to improve bovine semen diluents by glycine addition have been made *(5, 12, 13)*, only recently have such diluents shown promise of improving the livability of bovine spermatozoa during storage *(2, 6)*.

The studies reported in this paper were undertaken to investigate the feasibility of using combinations of glycine and skimmilk as a diluent for bovine semen. In much of this work, nonfat dry milk solids was employed in an effort to develop a diluent that could be stored in dry form for long periods and that would support satisfactory spermatozoan livability and fertility after reconstitution.

EXPERIMENTAL PROCEDURE

The Instant Pet nonfat dry milk solids (NFDMS) used in these trials was purchased from local markets. Reconstitution was at the rate of 8.7 g. of NFDMS per 100 ml. of water. Each lot of NFDMS was tested in an incubation trial and was used in the experiment only if the spermatozoa were immotile after 2 hours of incubation at 37° C. If the lot passed this qualifying test, it was detoxified for use as a diluent by heating at 92° C. for 10 minutes *(9)* or by adding 1 mg. of cysteine hydrochloride per milliliter of milk *(3)*. In the trials in which fresh liquid skimmilk was used, it was prepared in the laboratory by centrifugation of pasteurized whole milk. This procedure was deemed advisable in view of the unknown composition of commercial skimmilk *(7)*. In all experiments except Experiment 1, glycine was made up in stock solution of 0.5 *M* in distilled

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water. A milk glycine connotation implies a mixture of 1 volume of detoxified milk and 1 volume of 0.5 *M* glycine.

Semen was diluted to 15×10^6 spermatozoa per milliliter, cooled slowly to 4° C., and stored at that temperature. No antibiotics were added. Motility estimations over the 20-day storage period were made with a microscope equipped with a thermostage. Statistical analyses were conducted according to methods outlined by Snedecor *(8)*; all motility readings for the 20-day period were included in the analysis of variance.

RESULTS

Experiment 1. This experiment was performed in an attempt to determine the optimum concentration of glycine that might be combined with milk and to determine how much deviation from the optimum concentration could be tolerated. Glycine solutions of 0.0625, 0.125, 0.25, 0.5, and 1.0 *M* concentrations in distilled water were prepared, and each was mixed with an equal volume of reconstituted NFDMS. Livability of spermatozoa stored in these diluents is presented in Table 1. The optimum concentration of 0.5 *M* glycine gave significantly higher spermatozoan livability ($P < 0.01$) than either 0.25 or 1.0 *M*, indicating that the useful concentration range is rather narrow.

An exploratory trial involving six ejaculates was conducted to determine if the milk-glycine diluent might be improved by the addition of fructose, glycerol, or arginine. The treatments included *(a)* a control of reconstituted NFDMS, *(b)* NFDMS-0.5 *M* glycine, *(c)* *b* plus fructose to a final concentration of 0.2%, *(d)* *b* plus arginine to 0.2%, *(e)* *b* plus glycerol to 10%, and *(f)* egg yolk–0.5 *M* glycine (1:1). The egg yolk–glycine was superior to all other diluents tested in this trial; of the milk diluents only NFDMS-glycine-glycerol maintained livability significantly above that of the control.

Experiment 2. Since glycerol showed promise of improving livability in the exploratory trial just described, Experiment 2 was set up to compare different concentrations of glycerol in the NFDMS-glycine diluent. The data from this experiment are presented in Table 2. Of the glycerol levels tested, the 5% level supported livability to a significantly greater extent than did 0 or 10% glycerol. Livability in 2.5% glycerol approached that in 5% glycerol.

A sample of lipoprotein, prepared by the procedure of Kampschmidt *et al. (4)*, was included in this trial to determine if this fraction might account for the excellent results obtained with egg yolk–glycine in the exploratory trial. Lipoprotein appeared to be an excellent diluent for the first week of storage, but motility dropped suddenly after the eighth day. Motility estimations with this diluent were extremely difficult to perform and are probably unreliable owing to the fine emulsion formed by the lipoprotein in milk-glycine.

Experiment 3. In this experiment, three levels of glycerol (0, 2.5, and 5.0%) were tested in each of two media: fresh skimmilk–glycine and egg yolk–glycine (1:1). The results are summarized in Table 3. The improved livability previously observed when glycerol was added to reconstituted NFDMS-glycine was

TABLE 1

The effect of concentration of glycine when combined with heated NFDMS (1:1) (mean % motile spermatozoa in semen of 7 bulls)

	Days of storage at 4° C.											
	1	2	3	4	6	8	10	12	14	16	18	20
NFDMS	70	70	69	64	57	39	20	3	0	0		
NFDMS + 0.0625 *M* glycine (1:1)	27	26	20	20	17	17	16	10	7	3	0	0
NFDMS + 0.125 *M* glycine (1:1)	34	33	29	29	27	23	19	13	9	3	0	0
NFDMS + 0.250 *M* glycine (1:1)	59	59	57	54	41	37	30	17	9	6	4	0
NFDMS + 0.50 *M* glycine (1:1)	61	60	59	56	46	40	31	23	19	10	4	1
NFDMS + 1.00 *M* glycine (1:1)	56	51	51	51	37	26	14	9	6	4	0	0

TABLE 2

Effect of addition of glycerol or lipoprotein to cysteine-treated NFDMS-0.5 M *glycine (1:1) on spermatozoan livability (mean % motile spermatozoa in semen of 6 bulls)*

	Days of storage at 4° C.											
	1	2	3	4	6	8	10	12	14	16	18	20
NFDMS	68	68	68	65	53	33	17	5	3	2	0	0
NFDMS-glycine	65	65	65	58	38	25	7	5	5	2	2	0
NFDMS-glycine + 10% glycerol	65	65	62	57	42	37	23	17	7	3	3	2
NFDMS-glycine + 5% glycerol	68	68	65	60	45	42	27	20	15	10	8	5
NFDMS-glycine + 2.5% glycerol	67	67	62	62	47	40	30	17	10	10	8	3
NFDMS-glycine + 5% lipoprotein	68	68	67	65	58	50	17	0	0			

not apparent when fresh skimmilk was used. In the egg yolk–glycine series, motilities were significantly higher in 5.0% glycerol than in 0 or 2.5% glycerol ($P < 0.05$). Each of the skimmilk-glycine treatments enhanced livability to a significantly greater extent than did egg yolk–glycine with 0 or 2.5% glycerol ($P < 0.01$), but not egg yolk–glycine with 5% glycerol.

Spermatozoan survival was determined in this experiment and in Experiment 4 by observing semen samples daily the first 4 days and thereafter on alternate days until progressive motility ceased. Mean survival appeared to be considerably longer in egg yolk–glycine than in skimmilk-glycine treatments, although none of the survival data were subjected to statistical analysis.

Experiment 4. In this trial fresh skimmilk–glycine and reconstituted NFDMS-glycine were compared. Comparisons of heat and cysteine hydrochloride detoxification also were made on the fresh and reconstituted milks. As shown in Table 4, fresh skimmilk–glycine was significantly better than reconstituted NFDMS-glycine ($P < 0.01$) regardless of the method of detoxification. Only the fresh skimmilk (heated)–glycine supported livability superior to that in egg yolk–glycine ($P < 0.01$). Livability in NFDMS (cysteine)–glycine was superior to that in NFDMS(heated)–glycine ($P < 0.01$).

Experiment 5. A 4×4 factorial experiment was conducted in which four treatments, *(a)* NFDMS *(b)* NFDMS-glycine, *(c)* *b* plus glycerol to a final concentration of 5%, and *(d)* *b* plus fructose to a final concentration of 0.2%, were imposed on each of four lots of NFDMS. The "lots" were individual purchases, made at intervals of not less than 3 weeks, of a single brand of NFDMS from local markets. The results are presented in Table 5. Differences between lots were small. NFDMS-glycine maintained livability longer than did NFDMS alone. NFDMS-glycine-glycerol was superior to the other treatments ($P < 0.01$); NFDMS-glycine-fructose supported livability better than did either NFDMS-glycine or NFDMS alone ($P < 0.01$).

DISCUSSION

The addition of a 0.5 *M* aqueous glycine solution to skimmilk results in a diluent which sustains spermatozoan livability comparable to that in egg yolk–glycine. Results with combinations of reconstituted NFDMS-glycine were not as good as those obtained with fresh skimmilk–glycine (Table 4). However, the addition of glycerol and possibly of fructose to reconstituted NFDMS-glycine produced considerable improvement in results with the reconstituted NFDMS (Table 5).

Motility readings in milk-glycine often were lower than in milk alone during the first week of storage, but this relationship was reversed after the first week (*e.g.*, see Table 5). Although the over-all analysis under these conditions may indicate a difference in favor of milk-glycine, such an analysis may not be an accurate guide to performance in the field where semen is used normally within 2 or 3 days after collection. It is of interest to note that the addition of glycerol

TABLE 3

Effect of level of glycerol on spermatozoan livability in heated fresh skimmilk–0.5 M glycine and egg yolk–0.5 M glycine diluents (means obtained with semen of 11 bulls)

	Days of storage at 4° C.												
	1	2	3	4	6	8	10	12	14	16	18	20	Survival
						(% motility)							*(days)*
Skimmilk	58	57	54	49	43	21	9	3	0	0			11
Skimmilk-glycine	64	62	61	60	55	45	41	28	23	18	8	6	29
Skimmilk-glycine + 2.5% glycerol	64	63	62	61	55	46	41	27	22	20	9	8	32
Skimmilk-glycine + 5.0% glycerol	67	66	63	63	57	47	36	25	20	16	8	6	33
Egg yolk–glycine	63	55	53	53	51	41	29	24	23	18	15	14	44
Egg yolk–glycine + 2.5% glycerol	63	61	60	57	53	44	26	25	18	14	9	7	37
Egg yolk–glycine + 5.0% glycerol	62	62	60	57	55	45	30	24	24	20	11	9	41

TABLE 4

Effect of method of detoxification of milk on spermatozoan livability in fresh skimmilk-0.5 M glycine and NFDMS-0.5 M glycine diluents (means obtained with semen of 10 bulls)

	Days of storage at 4° C.												
	1	2	3	4	6	8	10	12	14	16	18	20	Survival
						(% motility)							*(days)*
Egg yolk–glycine (1:1)	66	64	62	56	51	46	34	29	24	18	17	15	37
NFDMS (heated)-glycine	67	67	64	56	40	28	19	17	11	0	0	0	15
NFDMS (cysteine)-glycine	61	61	60	56	47	42	31	28	19	5	3	2	21
Fresh skimmilk (heated)-glycine	67	66	64	61	57	52	46	43	36	23	14	6	26
Fresh skimmilk (cysteine)-glycine	61	61	61	57	53	47	39	32	25	19	15	7	25

TABLE 5

Effect of glycine, glycerol, fructose and lot of NFDMS on spermatozoan livability in milk diluents (mean % motile spermatozoa in semen of 6 bulls)

Lot of NFDMS	Days of storage at 4° C.									
	2	4	6	8	10	12	14	16	18	20
					NFDMS					
1	48	43	40	30	18	10	7	7	2	0
2	48	47	43	30	18	13	7	2	0	0
3	47	42	35	23	17	13	8	7	2	2
4	47	43	40	20	17	10	10	8	2	0
mean	48	44	40	26	18	12	8	6	1	0
					NFDMS-0.5 *M* glycine (1:1)					
1	43	40	38	33	28	23	20	13	10	7
2	42	37	33	30	25	17	12	5	5	3
3	45	37	35	30	25	22	18	12	8	5
4	42	38	37	28	27	22	20	15	8	5
mean	43	38	36	30	26	21	18	11	8	5
					NFDMS-0.5 *M* glycine (1:1) + 50% glycerol					
1	52	48	43	38	35	27	25	18	17	13
2	57	50	43	40	35	28	27	20	15	12
3	53	47	43	38	33	25	23	17	17	13
4	55	47	45	38	32	27	27	18	12	8
mean	54	48	44	39	34	27	25	18	15	12
					NFDMS-0.5% *M* glycine (1:1) + 0.2% fructose					
1	45	42	38	38	30	25	20	13	10	5
2	52	45	42	37	28	20	17	13	8	5
3	45	42	40	35	30	25	22	17	7	5
4	47	45	45	37	27	18	17	13	7	3
mean	47	43	41	37	29	22	19	14	8	5

tends to eliminate the low motility readings observed in milk-glycine during the first week of storage.

From the data presented it is apparent that glycine reduces motility during the first few days of storage at 5° C. but thereafter maintains motility over a considerably extended storage period. The reason for such an effect is not easily explained. Low levels of glycine, although beneficial to sea urchin spermatozoa, have not been shown to be of value for bovine spermatozoa *(12, 13)*. The beneficial action of glycine has been attributed to its metal-binding capacity *(11)*, but it seems unlikely that the relatively high concentration of glycine required in bovine semen diluents is required specifically for this function.

Protection against cold shock does not appear to be a factor, for semen samples subjected to cold shock prior to incubation at 37° C. have responded similarly whether diluted in milk-glycine or milk alone *(1)*. Unpublished data *(1)* indicate that fructolysis and lactic acid production are reduced in glycine-containing diluents; this may mean a reduction in the rate of metabolism or a shift from glycolytic processes. Studies with carbon 14–labeled glycine indicate that glycine is metabolized by spermatozoa *(1, 2)*, and the importance of this process is being investigated.

SUMMARY

A series of laboratory experiments was conducted in which livability of bovine spermatozoa was compared in combinations of aqueous solutions of glycine with *(a)* fresh skimmilk, *(b)* reconstituted nonfat dry milk solids (NFDMS), or *(c)* egg yolk. The effect of the addition of glycerol to these diluents also has been studied.

Each of five aqueous solutions of glycine (0.0625 to 1.0 *M*) was mixed with an equal volume of reconstituted NFDMS and the mixtures were tested as semen diluents. Based on this trial, the 0.5 *M* glycine solution was used in subsequent experiments.

Fresh skimmilk (heated)–0.5 *M* glycine (1:1) sustained livability equally as well as did egg yolk–0.5 *M* glycine (1:1), and significantly better than did heated skimmilk alone.

Mean livability over a 20-day storage period at 5° C. in reconstituted NFDMS–0.5 *M* glycine was superior to that in reconstituted NFDMS alone, and the addition of glycerol or fructose to the NFDMS–0.5 *M* glycine resulted in a further improvement in livability.

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STUDIES ON MILK FEVER IN DAIRY COWS. V. THE EFFECT OF MASSIVE ORAL DOSES OF VITAMIN D ON ABSORPTION, EXCRETION, RETENTION AND BLOOD LEVELS OF CALCIUM AND PHOSPHORUS[1]

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The efficiency of calcium absorption and mobilization is involved as a major contributory factor in the onset of milk fever. A study concerned with changes in the physiological behavior of calcium and phosphorus when massive doses of vitamin D are fed to dairy cattle assumes significant importance. Recent development of radioisotope procedures for the separate estimation of endogenous and exogenous fecal calcium and phosphorus provided the means for further study of this important problem. *Editor.*

This study is concerned with changes in the physiological behavior of calcium and phosphorus when massive oral doses of vitamin D are fed to cattle.

Recently Hibbs and Pounden *(13)* have shown that 30 million units per day of vitamin D fed 3-8 days prepartum prevented milk fever in mature cows with previous milk fever histories. The protection afforded by vitamin D was attributed to its calcemic effect, which was indicated by the relatively high blood calcium level maintained during the critical postpartum period. Other studies *(21)* have suggested that the increased blood calcium level was not due to parathyroid stimulation, because parathyroid activity was suppressed after 7 days of vitamin D feeding at thirty million units per day. In support of this, various workers *(1, 15)* using other species have found that calcemic doses of vitamin D increased absorption of calcium from the intestine, but parathyroid hormone secretion actually declined *(1)* and parathyroid size decreased *(16)*. The fact has been established experimentally in a number of species that vitamin D increases the absorption of calcium and phosphorus when added to a diet low in vitamin D *(6, 10, 17, 19)*. These findings suggest that the calcemic effect of vitamin D is elicited chiefly through an increased intestinal absorption of calcium and phosphorus.

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The marked diminution of calcium absorption with increased age in cattle *(8, 9)* and the characteristic occurrence of milk fever in aged cows as opposed to young cows *(11)* suggest that the efficiency of calcium absorption and mobilization is involved as a major contributory factor in the onset of milk fever. Ward *et al. (25)* found by conventional balance methods that aged cows which developed milk fever were in severe negative calcium balance for 15 days prepartum whereas others maintained their calcium stores, and that primiparous Jerseys appeared to store calcium, prepartum. The relation of age to calcium metabolism was indicated also by Moodie *et al. (18)*, who showed that the decline in serum calcium and rise in serum magnesium increased with age up to the fourth calving.

With the development of radioisotope procedures for the separate estimation of endogenous and exogenous fecal calcium and phosphorus *(9, 14, 22)*, an opportunity was presented for further study of the specific effects of vitamin D when fed prepartum to cattle in amounts sufficient to prevent milk fever.

EXPERIMENTAL PROCEDURE

This study involved 24 concurrent chemical and radiocalcium and phosphorus-32 balances with four lactating, two nonlactating cows (aged purebred or grade Jerseys), and two mature Hereford steers. All animals were oriented in metabolism units *(7)* following pre-experimental adjustment to rations of known composition.

In general three consecutive balances with each animal were carried out, including periods before, during, and after the feeding of 20 to 30 million units of vitamin D by capsule daily for five consecutive days. A viosterol-oil solution containing one million U.S.P. units per gram was used as a source of vitamin D. A single oral or intravenous tracer dose of radioactivity was administered to the animals by methods previously described *(9)* on the first day of the balance period. In the first phase of this study tracer doses of radiocalcium chloride were administered orally and intravenously, respectively, to paired animals on adequate rations, and periodic blood samples were taken throughout the 5- to 7-day balance trials. The procedure was then repeated several weeks later with phosphorus-32 in similar trials with two of the animals.

Feed, feces, urine, and milk samples were obtained routinely, dried overnight at 100° C., and ashed at 625° C. in muffle furnaces for 18 hours. The dry ash was then dissolved in 6 *N* HCl, an aliquot was taken, and the calcium was precipitated as the oxalate after adjusting the pH. Phosphorus was determined colorimetrically by a modification of the method of Fiske and Subba-Row *(4)*. Analyses for radiocalcium were carried out as described previously *(2)* and for radiophosphorus by direct counting of the wet ashed samples. Total calcium, phosphorus, and radioactivity of the periodic blood samples were determined by conventional procedure *(9)*.

Endogenous fecal losses were determined by using concurrently the comparative method described by Hansard *et al. (9)* and the isotope dilution *(14, 22)* procedure modified to include a single tracer dose of radioactivity *(9)*.

RESULTS

Results of the calcium and phosphorus balances before, during, and after feeding massive oral doses of vitamin D are summarized in Tables 1 and 2. A pronounced rise in calcium absorption was observed during vitamin D feeding. The average true digestibility coefficients for calcium increased from 33.1 to 50.3 for nonlactating cows (Table 1). True digestibility of the total phosphorus was found also to increase during the 5-day feeding period when 20 million units of vitamin D were fed daily to mature steers (Table 2). This increased true digestibility was accompanied by a marked decrease in endogenous calcium losses and an increase in net calcium retention (Table 1). A further increase in per cent true digestibility of both calcium and phosphorus occurred during the 5-day period immediately following vitamin D feeding.

The effects of vitamin D feeding upon fecal endogenous losses and retained calcium in the lactating cow were small with only a slight effect on net calcium retention. Thus it appeared that the vitamin D effect on calcium storage was most pronounced in dry cows. The lactating cows absorbed a greater percentage of their calcium intake than the nonlactating animals (Table 1).

TABLE 1

The effect of feeding massive oral doses of vitamin D[a] to mature cattle on the digestibility, excretion, and retention of total calcium and calcium[45]

	Nonlactating cattle[b]			Lactating cattle[c]		
	Before vitamin D feeding	During vitamin D feeding	After vitamin D feeding	Before vitamin D feeding	During vitamin D feeding	After vitamin D feeding
No. of animals	2	4	4	3	4	3
Vitamin D (*units/d.*)	none	30,000,000	none	none	30,000,000	none
Calcium intake (*g.*)	26.9	30.3	27.7	64.8	59.4	54.1
Fecal calcium (*g.*)	27.9	20.8	20.0	46.3	39.5	29.9
Urine calcium (*g.*)	0.1	0.9	0.5	0.2	0.6	0.3
Milk calcium (*g.*)	—	—	—	10.4	10.2	8.8
Calcium retained (*g.*)	–1.1	8.6	7.3	5.8	6.5	11.0
Ca. apparent digestibility (%)	–9.9	31.8	27.9	26.6	33.3	45.1
Fecal endogenous calcium (*g.*)	11.3	5.7	7.2	9.8	8.8	9.3
Ca. true digestibility (%)[d]	33.1	50.8	54.3	44.3	49.6	60.8
Ca^{45} excreted in feces (*% dose*)						
I.V. dose	19.6	18.8	21.7	—	17.7	—
Oral dose	80.7	78.2	53.9	—	53.7	—
Phos. apparent Digestibility (%)	9.9	44.1	41.9	27.4	45.5	48.8

[a] Viosterol. Administered daily by capsule.
[b] Two mature steers and three dry dairy cows used in this group.
[c] Four dairy cows in 5th-8th month of lactation used in this group.
[d] Differences between means due to vitamin D feeding significant at the 1% level of probability.

TABLE 2

The effect of feeding massive oral doses of vitamin D[a] to mature steers on the digestibility, excretion, and retention of total phosphorus

	Before vitamin D feeding	During vitamin D feeding	Directly after vitamin D feeding
No. of animals	2	4	2
Vitamin D (*units/d.*)	none	20,000,000	none
Phosphorus intake (*g/d.*)	30.3	27.9	26.0
Fecal phosphorus (*g/d.*)	29.2	21.9	14.8
Urine phosphorus (*g/d.*)	1.22	3.69	4.73
Phosphorus retained (*g/d.*)	−0.3	3.5	6.5
Phos. apparent digest. (%)	3.5	21.5	43.0
Fecal endogenous phos. (*g/d.*)	13.5	9.4	10.4
Phos. true digest. (%)	47.6	54.5	83.5
Total endogenous phos. loss (*g/d.*)	14.7	13.1	15.1

[a] Viosterol. Administered twice daily in capsules.

Although fecal endogenous phosphorus losses were decreased during the period of vitamin D feeding, a concomitant rise in total urine phosphorus approximately equaled this decrease; thus the net endogenous phosphorus losses for all animals were about the same for three feeding periods (Table 2).

The daily changes in calcium and phosphorus absorption and in blood levels are shown graphically in Figure 1. From the first day of vitamin D feeding there was an immediate rise in per cent of calcium and phosphorus intake absorbed, which persisted throughout the next 10 days of the test. On the other hand, total plasma calcium and inorganic phosphorus were not markedly affected until the fifth day of vitamin D feeding, at which time

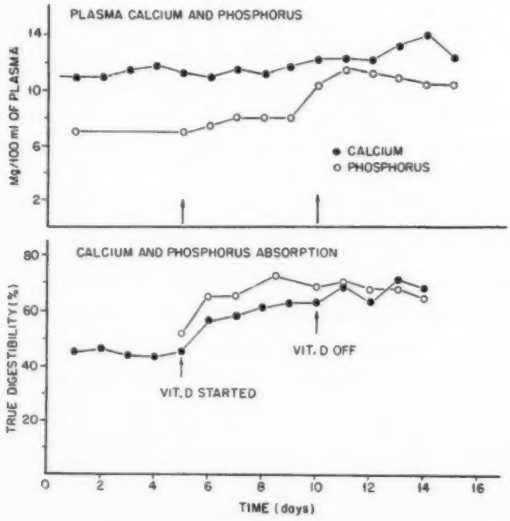

FIG. 1. Changes in true digestibility and blood levels of total calcium and phosphorus as a result of feeding mature steers 30 million units of vitamin D for 5 days.

the plasma calcium level began to increase slightly. This was accompanied by a marked increase in plasma inorganic phosphorus (Figure 1). It is noteworthy that both the per cent intake absorbed and the blood calcium and phosphorus levels remained at a maximum for the 5-day period following vitamin D feeding.

The increase in true digestibility of calcium was accompanied by an increase in radiocalcium concentration in the blood of nonlactating cattle dosed orally with Ca-45, whereas blood radiocalcium was approximately the same in the lactating cows receiving vitamin D as that of the control group (Figure 2).

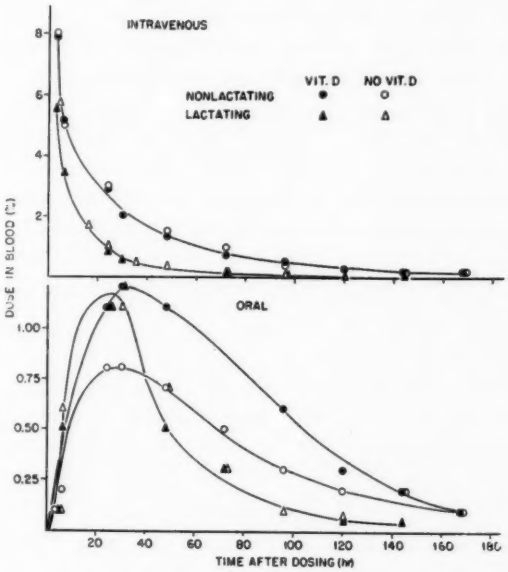

FIG. 2. The per cent of orally and intravenously administered radiocalcium in the blood as a function of time after dosing. Two lactating and two nonlactating cows included in each group with and without vitamin D.

Blood radiocalcium concentration was not affected by vitamin D feeding in the animals receiving Ca-45 intravenously. However, radiocalcium disappearance rate from the blood appeared to be more rapid in the lactating than nonlactating cows, even though their average age was approximately the same (Figure 2).

Analysis of these curves in Figure 2 by the methods of Flexner *et al. (5)* and Thomas *et al. (21)*, as outlined by Hansard *et al. (8)* for determining the rate of exchange of calcium between the plasma and other metabolic pools, showed that 39% of the calcium disappeared from the blood per minute in the nonlactating cows whereas in lactating cows this rate was calculated to be 54% per minute.

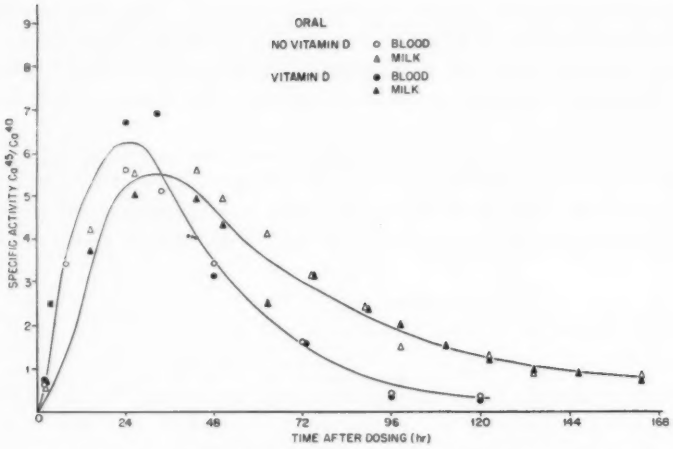

FIG. 3. The specific activity $\left(\frac{Ca^{45} \times 10^4}{Ca^{40}}\right)$ of blood and milk in relation to time after administering radiocalcium orally in a single pair of cows with and without vitamin D.

Observations on the average specific activity of the blood and milk from two cows receiving vitamin D, corrected for average time of secretion, are plotted graphically in Figure 3 with similar results from other cows. These limited data indicate that vitamin D did not affect the proportion of Ca-45 to Ca-40 secreted in the milk. A lag of approximately 12 hours was observed in the peak specific activities between the blood and milk curves, and the specific activity of the milk calcium remained higher than that of the blood throughout the remainder of the collection period. The rates of decline of both blood and milk values are exponential and they are essentially parallel.

DISCUSSION

The principal fundamental action of feeding massive oral doses of vitamin D appears to be an increase in the rate and quantity of calcium and phosphorus absorbed from the digestive tract. This effect was accompanied by a decreased excretion of these minerals into the intestinal tract and thus necessarily an increased net retention of both calcium and phosphorus. In subsequent experiments with 6-month-old calves sacrificed 7 days after dose administration tissue distribution studies showed Ca-45 concentration to be higher in the trabecular bone of the vitamin D fed calves than in that of their controls *(3)*. That the decreased endogenous loss is a secondary effect, perhaps caused by the increased movement of calcium and phosphorus from the gastrointestinal tract into the blood, is suggested by the fact that total blood calcium increased slightly during vitamin D feeding (Figure 1). This increase has been found to be due primarily to an increase in ionic calcium *(12)*.

Vitamin D feeding caused only a slight increase in plasma calcium levels, whereas the increase in net retention of calcium resulting from increased absorption was marked. Thus, it is apparent that the homeostatic mechanism regulating blood calcium in cattle was not seriously overwhelmed by the feeding of twenty to thirty million units of vitamin D for a 5-day period. These results suggest further that the massive doses of vitamin D employed by Hibbs and Pounden *(13)* for prevention of milk fever had no adverse physiological effects upon the bone storage mechanisms but rather augmented calcium reserves which could be drawn upon during the critical postpartum period. Evidence that prepartum enhancement of calcium reserves is a sound approach to prevention of milk fever is found in the work of Ward *et al. (25)*. They showed that cows which had milk fever were in greater negative calcium balance for the 15 days preceding parturition than normally freshening cows.

The per cent true digestibility of calcium and phosphorus increased during the 5-day period immediately following vitamin D feeding. This may have been due in part to a time lag in passage through the digestive tract and/or to the residual effects of absorbed vitamin D. Although increased absorption was maintained throughout this period, it is known from previous experience that preventive action of vitamin D for milk fever is limited to about 2 days or less after feeding is terminated. This suggests that vitamin D in preventing milk fever may have an additional effect other than increased mineral absorption from the gastrointestinal tract. The increased calcium retention observed in these experiments indicated that mineralization of the bone was taking place, possibly associated with low parathyroid hormone activity during vitamin D feeding.

Early studies with massive doses of vitamin D have demonstrated an increase in the urinary excretion of phosphates *(1)*. This is considered to be a separate action independent of increased gastrointestinal absorption. It has been shown by these studies that feeding 20 to 30 million units of vitamin D per day for a 5-day period markedly increased urinary phosphate excretion in mature cattle. The next 5-day period following the termination of the vitamin D feeding was characterized by *(a)* high plasma inorganic phosphate levels, *(b)* high plasma calcium, *(c)* further increase in urinary phosphates, and *(d)* a tendency for the endogenous fecal losses of calcium and phosphorus to return toward the pre-vitamin D level. These observations suggest that following vitamin D feeding, mobilization of the calcium was occurring. This may indicate return of parathyroid activity.

The increased rate for the disappearance of radiocalcium (Figure 2) from the blood of the lactating cows (54%) compared to nonlactating cows (39%) is interpreted as being indicative of the increased rate of calcium movement through the plasma caused by secretion into the milk. In this experiment the lactating cows were secreting in the milk an average of 10 g. per day; therefore, a greater rate of calcium movement would be expected (Table 1). The lag in the peak specific activity of the milk calcium and its higher specific

activity during subsequent sampling periods is in agreement with similar results reported and discussed by Visek *et al. (23)* and Swanson *et al. (20)*. This observation is indicative of a variable calcium reserve in the udder or a variation in the exchangeability of stored calcium with plasma calcium.

SUMMARY AND CONCLUSIONS

Tracer doses of Ca-45 and P-32 were used as markers in balance trials and blood studies with mature lactating and nonlactating cattle to evaluate the changes in absorption, excretion, and retention of these minerals when massive doses of vitamin D were given.

These studies show that massive oral doses of vitamin D increased calcium and phosphorus absorption from the digestive tract. There was a decrease in endogenous losses accompanied by an increase in net retention, which extended throughout a 5-day period following vitamin D feeding. These changes in the absorption pattern for calcium and phosphorus were accompanied by a delayed increase in blood levels of calcium and phosphorus. A rise in blood phosphorus was observed to precede the rise in blood calcium. The increase in net absorption of total calcium by nonlactating cattle was accompanied by an increase in radiocalcium concentration in the blood. Radiocalcium disappeared from the blood of lactating cows at a faster rate than from nonlactating cows. Vitamin D was without effect on specific activity of milk calcium.

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THE COMPOSITION OF HOLOCELLULOSE PREPARED FROM VARIOUS FORAGES AND THE CORRESPONDING FECES

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The lignin, protein, ash, and carbohydrate contents of crude holocellulose samples prepared from ten common forages and from the feces of dairy cows fed these forages were determined. *Editor.*

The composition of holocellulose prepared from corn silage *(9)*, alfalfa silage *(9)*, timothy hay *(4)*, mixed hay *(5)*, oat hay, *(5)*, bluegrass *(4)*, orchard grass *(9)*, cornstalks *(4, 5, 6)*, straw *(1, 2, 4, 9, 10)* and corn cobs *(4, 11)* has been reported. Holocellulose samples prepared from the above materials contained residual lignin and protein. Comparing the composition of holocellulose from these materials is difficult since different methods of preparation and varying lengths of treatment were used.

Wise *et al. (13)* found that holocellulose from wood was very low in nitrogen when prepared by the acid chlorite method. Most wood, however, contains very small amounts of nitrogen before delignification. Reports of the lignin content of holocellulose prepared from wood are too numerous to review here and are adequately discussed by Wise and Jahn *(12)*. Coldwell and DeLong *(7)* found that two or three acid chlorite treatments removed from 74.4 to 84.0% of the protein and from 77.2 to 87.1% of the lignin from extracted leaves of beech, birch, maple, and poplar trees. The authors are not aware of any reports on the composition of holocellulose prepared from ruminant feces.

The object of this investigation was to study the lignin, protein ($N \times 6.25$), ash, and carbohydrate contents of crude holocellulose samples prepared from a variety of common forages and from the feces of dairy cows fed these forages.

EXPERIMENTAL PROCEDURE

Ten forages were chosen that varied greatly in the amounts of lignin and the constituents of the proximate analysis. Silage, hay, and straw were included in the forages studied. Five grasses and five legume species were represented. Each of these forages was fed to one dairy cow as the entire ration. After a preliminary feeding period of 15 days, fecal samples were collected four times daily for three consecutive days from each animal. The collections were dried in enamel trays placed in a circulating air sample dryer operating

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at 60-70° C. The dried samples for the three collection days were composited and ground through a medium screen Wiley mill for subsequent analyses.

Holocellulose samples were prepared from the extractive-free *(9)* forage and feces samples by the procedure of Wise, Murphy, and D'Addieco *(12)* with minor modifications. Six 1-g. samples were prepared of each material and two determinations were made for ash-free crude holocellulose, lignin, and nitrogen contents. Lignin determinations were made by the method of Ellis, Matrone, and Maynard *(8)*. Nitrogen was determined by the conventional Kjeldahl procedure *(3)*, and the protein content was calculated on the basis that it contained 16% of nitrogen. Ash was determined by ignition at 600° C. The carbohydrate content of the ash-free crude holocellulose was calculated as the protein and lignin-free organic matter.

RESULTS AND DISCUSSION

The proximate compositions of the forage and feces samples used for the preparation of holocellulose are presented in Table 1. The protein content of the forage ranged from 3.2 to 22.0% and the lignin content from 5.5 to 13.5%. The protein content of the feces ranged from 6.0 to 20.0% and the lignin content from 16.5 to 30.9%. The highest and lowest contents of protein in the feces were from the animals fed the high and low protein forage, respectively. The same relationship does not exist between the lignin content of forage and the lignin content of the corresponding feces.

The composition of the holocellulose samples prepared from the forages fed and from the corresponding feces is presented in Table 2. The contents of protein, lignin, and carbohydrates are given as the percentage of these constituents in the ash-free holocellulose since the crude holocellulose is routinely determined as ash-free material.

The protein contents of holocellulose samples prepared from feces were higher than the protein contents of holocellulose samples prepared from the forages fed with seven of the ten comparisons. The lignin contents of holocellulose samples prepared from feces were higher than the lignin contents of the holocellulose prepared from the corresponding forages in all of the comparisons studied. The lignin contents were lower in each of the forages than in the corresponding feces used for preparing the holocellulose samples (Table 1).

The ash-free holocellulose samples prepared from the ten forages contained from 84.5 to 97.4% of carbohydrates. This calculation assumes that the protein and lignin-free organic matter of the crude holocellulose is entirely carbohydrate material. The ash-free holocellulose from the ten feces samples contained from 81.6 to 94.2% of carbohydrates. The crude holocellulose samples prepared from the forages of lower protein content and the corresponding feces had higher percentages of carbohydrates than the crude holocellulose samples prepared from the forages of higher protein content and the corresponding feces.

TABLE 1

Per cent composition of dry matter of forage and feces

Forage	Animal	Protein		Ether extract		Crude fiber		N.F.E.		Ash		Lignin	
		Forage	Feces	Forage	Feces	Forage	Feces	Forage	Feces	Forage	Feces	Forage	Feces
Wheat straw	X65	3.2	6.0	1.4	1.5	42.4	29.8	48.1	39.9	4.9	22.8	13.0	16.5
Timothy hay	N666	6.7	7.3	1.9	2.5	34.2	33.5	52.9	44.6	4.3	12.1	10.7	24.0
Corn silage	N645	7.4	12.0	2.3	1.7	22.7	27.3	63.2	48.4	4.4	10.6	6.9	22.6
Clover hay	N291	10.7	12.1	1.7	1.9	37.8	40.3	43.6	37.9	6.2	7.8	12.7	26.7
Mature orchard grass	N833	11.1	9.5	2.3	3.9	35.1	33.4	46.3	46.8	5.2	6.4	9.3	21.7
Soybean hay	N295	11.0	10.3	0.9	2.4	42.9	37.8	36.7	31.3	8.5	18.2	13.5	24.3
Lespedeza hay	N335	13.8	14.4	3.0	4.5	30.5	31.0	45.5	40.3	7.2	9.8	12.6	30.9
Alfalfa hay	N689	14.5	11.1	1.5	3.8	31.7	40.5	45.0	31.3	7.3	13.3	10.0	23.4
Alfalfa silage	N685	17.8	15.3	3.0	2.7	37.9	41.4	32.3	31.1	9.0	9.5	12.5	36.1
Young orchard grass	N622	22.0	20.0	3.0	8.1	24.1	21.4	44.4	41.4	5.8	9.1	5.5	19.5

In general, the crude holocellulose samples prepared from forages and feces in this study with the higher protein contents had higher percentages of both protein and lignin than holocellulose preparations from materials of lower protein content. The lignin and protein contents of holocellulose prepared from wheat straw in this study are similar to those reported by Adams and Castagne *(1)*. The authors recognize that conventional procedures for the determination of lignin are not completely reliable when applied to crude holocellulose.

The ash contents of holocellulose samples prepared from the ten forages and the corresponding feces are presented as supplemental information in Table 2. In general somewhat more ash was present in the crude holocellulose than was present in the material before acid chlorite treatment owing to retention of reagents by the holocellulose. All feces samples had higher ash contents than the forages fed. Crude holocellulose samples prepared from feces also had higher ash contents than the holocellulose samples prepared from the forages. The ash content of crude holocellulose should not be considered as a contaminant, since the content of crude holocellulose is routinely determined as ash-free material.

The true holocellulose content [ash-free crude holocellulose — (protein + lignin)] ranged from 39.0 to 74.6% of the forage dry matter and from 31.3 to 53.7% of the feces dry matter. Eight of the ten comparisons had higher contents of true holocellulose in the forage than in the feces dry matter.

These studies show that protein and lignin may be minor contaminants of crude holocellulose prepared from materials low in protein. On the other hand, protein and lignin may be contaminants of considerable magnitude in crude holocellulose prepared from materials high in protein. Protein is usually considered the principal contaminant of holocellulose prepared from forages. The present study shows that the lignin content is higher than the protein content in 40% of the holocellulose samples prepared from forage and in 80% of the holocellulose samples prepared from feces. The apparent lignin (material insoluble in 72% sulfuric acid) of holocellulose should be considered as a principal contaminant of many holocellulose samples, particularly those prepared from feces.

SUMMARY

The composition of holocellulose samples prepared from a variety of forages and from the feces of cows fed these forages were studied.

Holocellulose prepared from feces contained more lignin than holocellulose prepared from the forages.

The ash-free holocellulose prepared from ten forages contained from 84.5 to 97.4% of carbohydrates. The ash-free holocellulose prepared from the corresponding feces contained from 81.6 to 94.2% of carbohydrates.

Protein and lignin are minor contaminants of holocellulose prepared from materials low in protein.

TABLE 2

Per cent composition of crude holocellulose from forage and feces and the true holocellulose contents

Forage	Animal	Protein[a]		Lignin[a]		Carbohydrate[a]		Ash[b]		True holocellulose[c]	
		Forage	Feces	Forage	Feces	Forage	Feces	Forage	Feces	Forage	Feces
Wheat straw	X65	0.7	3.8	1.9	2.8	97.4	93.4	4.8	20.0	74.6	45.0
Timothy hay	N666	2.5	2.0	2.8	3.8	94.7	94.2	2.9	14.5	58.1	48.5
Corn silage	N645	1.8	4.7	1.6	3.7	96.6	91.6	3.4	9.4	42.4	44.1
Clover hay	N291	5.0	4.2	4.5	6.2	90.5	89.6	3.3	5.6	52.7	51.3
Mature orchard grass	N833	4.6	2.8	3.0	4.5	92.4	92.7	2.6	5.2	59.1	53.7
Soybean hay	N295	3.6	3.0	6.1	6.9	90.3	90.1	4.6	16.3	53.0	40.3
Lespedeza hay	N335	7.1	5.8	5.4	8.2	87.5	86.0	4.6	8.8	46.7	41.2
Alfalfa hay	N689	7.8	3.3	6.6	7.1	85.6	89.6	3.4	9.0	39.0	47.7
Alfalfa silage	N685	3.6	5.4	5.4	6.4	91.0	88.2	5.1	7.0	49.3	45.4
Young orchard grass	N622	11.1	8.6	4.4	9.8	84.5	81.6	4.3	7.7	44.2	31.3

[a] Per cent of ash-free crude holocellulose.
[b] Per cent of dry weight of crude holocellulose.
[c] Per cent of ash, protein and lignin-free holocellulose in forage and feces dry matter.

The protein and apparent lignin contents of crude holocellulose both increase when preparations are made from high protein materials.

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BULL AND LINE DIFFERENCES IN THE SURVIVAL OF SPERMATOZOA AFTER FREEZING AND THAWING[1]

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The survivability of bull spermatozoa, frozen and thawed under uniform conditions, was determined within lines, among lines, and at different periods. Twelve Holstein-Friesian bulls representing six inbred lines were used in the study. A marked difference among bulls in the ability of their spermatozoa to withstand freezing was noted though no evidence of genetic difference was indicated by the data obtained. *Editor.*

Numerous studies have been made on factors affecting the survival of bull spermatozoa after freezing at low temperatures *(2, 4, 5, 7, 8, 9, 11)*. No studies have been reported comparing the ability of spermatozoa from different bulls to survive freezing and thawing with a uniform procedure.

This study was undertaken to compare the survivability of spermatozoa frozen and thawed under uniform conditions from bulls within lines, between lines, and at different periods.

EXPERIMENTAL METHODS

Semen was collected once a week for 6 weeks from 12 Holstein-Friesian bulls representing six inbred lines maintained at the Emmons Blaine, Jr., Experimental Farm at Lake Mills, Wisconsin. Two ejaculates were taken from each bull at the weekly collection, but only the second ejaculate was used for this study. Each weekly collection was considered a batch. The six inbred lines were represented by two bulls for each line.

The range of inbreeding coefficients of the bulls used in this study was from 0.13 to 0.44; the average was 0.25. The coefficients of relationship between

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two bulls of the same line varied from 0.28 to 0.57, but in most cases the relationship approached that of full brothers. The coefficients of relationship between two bulls of different lines were practically nil.

Immediately after collection, the semen was partially diluted 1:5 with a diluter containing 25% of egg yolk (by volume) in 2.9% sodium citrate at 37° C. After the initial dilution, the semen was cooled slowly to 5° C. While samples of the partially diluted semen were cooling, the spermatozoa concentration of the undiluted semen was determined by means of turbidity *(3, 10, 14)*.

After the concentration of spermatozoa had been determined, the partially diluted semen samples were further diluted to half the final volume with the egg yolk–citrate diluter. About 6 hours after the initial dilution, an equal volume of egg yolk–citrate diluter containing 15% glycerol by volume was added in five aliquots at 15-minute intervals. The final concentration of glycerol was 7.5% and each milliliter of diluted semen contained about 20 million spermatozoa. The spermatozoa were allowed to equilibrate at a temperature of 5° C. for 18 hours before freezing. One ml. of the diluted semen was put into Pyrex glass tubes, which were cork-stoppered for freezing.

The rate of temperature decrease was 1° C. for every 3 minutes from 5° C. to –10° C., 1° C. per minute from –10° C. to –15° C., and as rapidly as possible from –15° C. to –75° C. The frozen semen was stored in trays in a mechanical freezer at –89° C. Arrangement of the tubes in the trays was at random and coded so that no bias could be introduced while estimating the per cent survival of spermatozoa.

The semen was thawed at 7° C. to 10° C. Motility estimations were made from two tubes of each sample, averaged and converted to angles for statistical analyses.

The analysis of variance of the orthogonal factorial design was made as suggested by Henderson *(6)*, considering the periods as the fixed variables and the rest of the variables as random variables. Different error terms were used to test the significance of difference among variables depending on the variance components of the mean squares. Special error terms and degrees of freedom were computed to test the significance of differences among periods and among lines *(1, 13)*.

Comparisons of the means of per cent survival of spermatozoa were made between *(a)* freshly diluted and the equilibrated semen, *(b)* between the equilibrated and 0-day post-freezing, and *(c)* between 0, 1, 3, 7, 14, 28, 56, and 84 days post-freezing.

RESULTS AND DISCUSSION

The mean per cent of motile spermatozoa after dilution, equilibration, and at various intervals post-freezing are presented in Table 1. Table 2 shows the test of significance at different pre- and post-freezing periods.

The difference of 8% motile spermatozoa between dilution and equilibration was significant ($P < 0.01$). There were no significant differences among lines

TABLE 1

Estimated per cent of motile spermatozoa after various treatments

Bull lines	Bulls within lines	Post-dilution	Post-equilibration	Days post-freezing							
				0	1	3	7	14	28	56	84
O	1	67	57	35	24	24	25	24	24	19	18
	2	60	47	15	13	14	13	8	8	8	7
	Av.	64	52	25	18	19	19	16	16	13	12
L	1	73	60	48	47	41	38	33	35	30	30
	2	69	60	43	43	41	38	36	33	30	24
	Av.	71	60	45	45	41	38	34	34	30	27
H	1	62	59	28	24	17	19	15	14	9	9
	2	60	57	31	28	22	24	18	15	15	12
	Av.	61	58	29	26	19	21	16	14	12	10
B	1	72	66	52	41	41	35	28	25	19	18
	2	73	70	54	54	48	41	45	41	33	33
	Av.	72	68	53	47	44	38	36	33	26	25
M	1	75	66	50	43	40	36	31	28	25	28
	2	67	60	41	41	38	36	36	27	24	25
	Av.	71	63	45	42	39	36	33	27	24	26
N	1	52	40	1	0	0	0	0	1	0	0
	2	75	66	40	38	36	33	25	24	21	21
	Av.	63	53	20	19	18	16	12	12	10	10
Mean—all bulls		67	59	33	31	28	27	22	20	18	17

TABLE 2

Tests of significance

Sources of variation	Post dilution and Post equilibration	Post equilibration and 0-day post-freezing	Post-freezing intervals
	(Prob.)	(Prob.)	(Prob.)
Among periods	< 0.01	< 0.01	< 0.01
Among lines	N.S.[a]	N.S.	N.S.
Among bulls within lines	N.S.	< 0.01	< 0.01
Periods × lines	N.S.	N.S.	N.S.
Periods × bulls within lines	N.S.	< 0.01	< 0.01

[a] N.S. indicates a probability greater than 0.05.

or among bulls within lines for these two periods. The average per cent of spermatozoa showing motility after equilibration was 59 as compared to 33 for 0-day post-freezing. This difference of 26% was highly significant ($P < 0.01$). The results at 0-day post-freezing are very close to the 39.7% survival obtained by Saroff and Mixner *(11)* when they used egg yolk and glycerol levels at 23% and 8%, respectively, with an equilibration time of 18 hours for semen from Holstein and Guernsey bulls regularly used in artificial breeding. Hafs and Elliott *(5)*, using seven Holstein bulls, observed that the average per cent of motile spermatozoa immediately after freezing was 30.5 when the spermatozoa were allowed to equilibrate with glycerol for 18 hours before freezing. Miller and VanDemark *(8)* reported that the mean per cent of motile spermatozoa was 34 and 35, respectively, when glycerol was used at 6 and 8% levels.

The range in the drop of motility at 0-day post-freezing was from 12% for L_1 bull to 39% for N_1 bull. The differences among lines in per cent motility for these pre- and post-freezing periods were not significant. A highly significant difference among bulls within lines and a highly significant interaction among periods and bulls within lines are suggestive of the differences in the ability of spermatozoa of different bulls to survive the process of freezing and thawing. It also suggests more pronounced differences among bulls and a differential response of bulls at different freezing periods. No attempt was made to alter the procedure to determine whether the per cent revival could be increased in the semen of those bulls with low survivability.

The average means of motile spermatozoa at different periods post-freezing show a significant decline in motile cells of 6% from 0 to 7 days, 5% from 7 to 14 days, 2% from 14 to 28 days, a further decline of 2% from 28 to 56 days, and an additional drop of 1% from 56 to 84 days. This difference among periods is highly significant ($P < 0.01$). The greatest decline in per cent of motile spermatozoa occurs during the first 14 days of storage.

In the majority of the semen samples the percentages of motile spermatozoa at 0- and 1-day post-freezing were similar, which is in agreement with the results obtained by Miller and VanDemark *(8)*, who observed that the samples stored for 1 hour exhibited approximately the same motility as did the samples stored for 1 day.

The differences among lines in the post-freezing periods were not significant, and as such this study does not indicate any evidence of genetic difference in the survival of spermatozoa at any time before or after freezing.

Although a few of the bulls show unusual changes in the survivability of spermatozoa after freezing, the trend for the majority of the bulls suggests that the survivability of spermatozoa after 84 days of storage could be predicted from an estimate of the survivability immediately following freezing and thawing. The correlation between the percentage of motile spermatozoa immediately following freezing and thawing and the percentage alive after 84 days of storage was 0.97 among bulls and 0.44 within bulls ($P < 0.01$). The corresponding regressions of 84-day on 0-day percentages were 0.73 and 0.47. The individual bull regressions were not heterogeneous.

SUMMARY

A study of the differences among bulls, among different inbred lines, and among different periods in the survivability of spermatozoa was made before and after freezing with 12 Holstein-Friesian bulls representing six inbred lines.

A marked difference was observed among bulls in the ability of their spermatozoa to withstand freezing, although there were no significant differences prior to freezing. No line differences were observed at any of the pre- or post-freezing periods and as such this study does not indicate any evidence of genetic difference in the survival of spermatozoa before or after freezing. Although the rate of decrease in motility of spermatozoa with time in storage at the temperature

used in this study differed among bulls, the per cent of motile spermatozoa after 12 weeks storage generally could be predicted immediately after freezing. A highly significant decline in the per cent survival of spermatozoa was observed from period to period. The rate of decline was the highest during the first two weeks after freezing.

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THE EXPERIMENTAL INDUCTION OF GROWTH OF THE COW'S UDDER AND THE INITIATION OF MILK SECRETION[1, 2]

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The purpose of this study was to determine the optimum amounts of two ovarian hormones and their synergism for growth of the udders of heifers that had failed to conceive. Maximum daily milk production attained when lactation was initiated by means of the estrogenic hormone was used as the index of udder growth. The udder growth and milk secretion that was stimulated closely approached that which might have been obtained had the heifers calved normally. Practical use of this method of inducing growth and lactation will be dependent upon the development of methods of oral administration of the hormones. *Editor.*

As a result of extensive research with experimental animals, it has been shown that the ovarian hormone, estrogen, will stimulate the growth of the mammary gland duct system of gonadectomized animals comparable to that observed in sexually mature animals *(28)*. However, to stimulate the growth of the lobule-alveolar system comparable to that observed at the end of pseudopregnancy or the middle of pregnancy, it was found necessary to inject a combination of estrogen and progesterone *(28)*. In recent work with crystalline ovarian hormones, the importance of the proper ratio of these two hormones for optimal growth of the lobule-alveolar system has been pointed out *(28)*.

In the rabbit, Scharf and Lyons *(18)* observed optimal synergism when 24 to 96 γ of estrone were administered with 1 mg. of progesterone daily for 25 days. This represents a ratio of estrogen to progesterone of 1:11 to 42. Yamamoto and Turner *(28)* recently confirmed their observations. However, in other experimental animals, the ratio of the two hormones for optimal synergism was observed to be of the order of 1 part of estrogen to 1,000 or more parts of progesterone, as shown in the mouse *(4, 19, 27)*; rat *(4)*, and dog *(21)*.

In spite of the extensive research work indicating the synergism of the two ovarian hormones for complete lobule alveolar growth of the mammary gland of experimental animals, comparable studies with goats have led to a variety of conclusions in regard to the need of progesterone for lobule-alveolar growth. Mixner and Turner *(12)* reported that the daily injection of 20-30 mg. of

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progesterone plus 100-150 γ of diethylstilbestrol for 60 days showed great development of tightly packed alveoli comparable to the glands of goats at midpregnancy. This is a synergistic ratio of 1:200 of estrogen to progesterone. At the end of 60 days the treatment was stopped and 0.25 mg. of diethylstilbestrol alone was injected for 12 days. Examination of the glands indicated the initiation of secretion with great enlargement of the alveoli comparable to that observed at parturition.

Cowie *et al. (3)* reported the results of injecting daily 1 mg. hexestrol and 40 mg. of progesterone for 70 or 140 days. It was suggested that increasing the treatment period to 140 days raised the yields appreciably above the 70-day level if progesterone was given and lowered them if it was not. It should be noted that the actual level of hexestrol was relatively very high and the ratio of the two hormones 1:40 was much narrower than that used by Mixner and Turner. Quantitative histological measurements failed to indicate any significant differences between glands treated with estrogen alone or by estrogen and progesterone.

In a subsequent study by the English group *(2)*, the daily injection of 0.5 mg. hexestrol plus 70 mg. progesterone for 150 days induced the growth of udders which were virtually free from histological abnormalities and in which the alveolar tissue was homogenous and compact.

In dairy calves (age 6 months) Sykes and Wrenn *(20)* compared the mammary glands after 5 months of injection of 6 mg. of diethylstilbestrol and 240 mg. of progesterone per week (ratio of 1:40) with those of calves given diethylstilbestrol alone. They concluded that diethylstilbestrol alone produced abnormal glands whereas the two hormones resulted in more nearly normal development.

At Michigan State University reports *(8, 9, 16)* on milk production induced in heifers and cows implanted with 100 mg. to 2 g. diethylstilbestrol and up to 4 g. of progesterone (ratio 1:2) indicate growth promotion by this treatment. However, it is difficult to estimate the daily rate of absorption from implanted pellets. Consequently, neither the daily effective dose nor ratio of the two hormones is known.

Hancock *et al. (5)* injected diethylstilbestrol and progesterone during a period of 5 months to one of a pair of identical twins. The two hormones were injected at a ratio of 1 γ of diethylstilbestrol to 1 mg. of progesterone (ratio 1:1000). These hormones were injected in increasing amounts starting with 50 mg. of progesterone and 50 γ of diethylstilbestrol for 2 months, 75 mg. and 75 γ for the third month and 100 mg. and 100 γ for the final 2 months. This treatment was then stopped and lactation was induced with diethylstilbestrol alone. Milk production was compared with that of the normal twin after parturition. Three of the seven sets of twins were very low producers. Experimental milk production was 10.8, 83.7, and 106.9% of the lactation of the normal twin. The other four higher producing animals yielded 41.9, 49.1, 61.1, and 72.5% of the milk of the normal mate.

Coincident with the present study Williams *et al. (26)* conducted a similar study using both sterile heifers and multiparous cows. Of a group of eight

Jerseys, seven reached a maximum yield varying from 16 to 29 lb. of milk. Details of the study have not been published.

The present study is an extension of the work begun in New Zealand to determine the optimum amounts of the two ovarian hormones and their synergism for growth of the udder of heifers. The heifers available for the research reported here were chiefly animals that had failed to conceive in the dairy herds of the Experiment Station. The high value of the individual animals precluded their sacrifice for udder examination at the end of the hormonal treatment. Instead, it was decided to use as an index of udder growth, the maximum daily milk production attained when lactation was initiated by means of the estrogenic hormone after the period of growth stimulation.

Such a lactation index of mammary gland growth involves the assumption that the number of epithelial cells present is directly related to the yield of milk. This is not a true relationship since cows might have the same number of gland cells yet differ in maximum milk production owing to variation in the rate of secretion of the lactogenic, thyrotropic, growth, and other hormones influencing the intensity of lactation. Some of these variations due to other hormonal secretion rates have been estimated by replacement therapy with these hormones. In spite of these limitations in the method of estimating the type and extent of udder development induced, it appears to be the best method available for our study.

Plan of the experiment. In outline, the plan of treatment of the heifers involved the daily injection of estradiol benzoate and progesterone together for a period of 180 days to attempt to stimulate the growth of the complete lobule-alveolar system similar to that developed during a comparable period of normal pregnancy. This treatment is then discontinued and a higher level of estradiol benzoate alone is administered for a period of 2 weeks to initiate lactation comparable to that induced at parturition.

Ratio of estrogen to progesterone. In the development of the above plan, the synergistic ratio of the ovarian hormones to use was first considered. Observations at this station with experimental animals indicated that a wider ratio than previously used should be employed with cattle. The use of narrow ratios in goats by the English investigators produced abnormal alveolar development (initiation of lactation). It was decided to try a ratio of at least 1:1000 of estrogen to progesterone.

The level of progesterone to use was based upon the requirements of progesterone to maintain pregnancy in dairy heifers from which the corpora lutea had been removed. The observations of Raeside and Turner *(14)*, Uren and Raeside *(25)*, and McDonald *et al. (7)* indicated that 100 mg. of progesterone daily would accomplish this objective. It was shown by Raeside and Turner *(15)* that detectable amounts of progesterone are present in the blood within ½ hour after injection but disappear in less than 4 hours unless the hormone is injected as microcrystals. It is possible that less than 100 mg. of progesterone daily would be adequate if injected in the latter form.

With 100 mg. of progesterone injected daily, the estrogen requirements on the basis of a ratio of 1:1000 would be 100 γ. It is believed that the dairy cow secretes a low level of estrogen, since Asdell *et al. (1)* induced a copulatory response in ovariectomized heifers with 100 γ of estradiol benzoate daily for 3 days. Since the urinary excretion of estrogen in the pregnant cow remains low during the first half to two-thirds of pregnancy *(23)*, these data provide further support for the suggested low estrogen requirement for synergism with progesterone for growth of the udder of cattle.

Time required to grow the mammary glands. In experimental animals, complete lobule-alveolar development requires from one-half to two-thirds of the normal period of pregnancy. An injection period of about 180 days was considered necessary to obtain complete growth.

Initiation of lactation. After the completion of the growth of the lobule-alveolar system during pregnancy, lactation is believed to be initiated by the gradual rise in the secretion of estrogen up to parturition. Estrogen has been shown to stimulate the increased secretion of the lactogenic hormone of the anterior pituitary *(10, 11)*, which in turn stimulates functional activity of the epithelial cells. Instead of gradually increasing the dosage of estrogen, as is believed to occur during the latter part of pregnancy, 3 mg. of estradiol benzoate daily was injected for a period of 14 days simultaneously with the withdrawal of progesterone.

Description of heifers. The present experiment included the following sterile animals: Jersey heifers No. 310, 580, 583, and 585 were obtained from the University herd along with Holstein heifer 402 and a Guernsey freemartin. In addition, two Jersey heifers, No. 160 and 544, were obtained from a local dairyman. One set of normal Jersey identical twins[3] was available, one of which was bred to calve normally (No. 158) whereas the second (No. 159) was induced to lactate experimentally (Table 1).

EXPERIMENTAL RESULTS

(a) Growth phase. For the first 180 days, the heifers were injected once daily subcutaneously with 1.2 ml. sesame oil containing a mixture of 100 mg. of progesterone and 100 γ of estradiol benzoate. Little or no change in the external appearance of the udders was observed during this period (Figure 1). This was to be expected since the ducts and lobule-alveolar systems invade the preformed fatty substance of the udder *(24)*. Slight lengthening of the teats usually occurred. The skin showed some evidence of increased hyperemia.

It is interesting to note that during the injection period the animals showed none of the undesirable features exhibited by animals injected with estrogen alone. Mounting and pelvic changes were not observed. Body weight gains were satisfactory. Estrous cycles were inhibited.

(b) Lactation phase. Upon the injection of 3 mg. daily of estradiol benzoate, the enlargement of the udder was initiated and continued until maximum daily

[3] See footnote to Table 1.

TABLE 1

Data on experimental cattle

Herd No.	Breed	Date of birth	Date at start of injections	Age at initiation of lactation (*mo.*)	Body weight		Remarks
					At start (*lb.*)	At end (*lb.*)	
310	Jersey	9/22/51	5/25/54	31	878	961	
580	Jersey	6/13/51	6/11/54	36	840	902	
402	Holstein	7/5/51	6/11/54	35	1,260	1,399	Twin to No. 401
583	Jersey	12/9/51	7/6/54	43	790	914	One blind teat
585	Jersey	4/24/52	7/6/54	25	825	922	
160	Jersey	5/30/50	2/7/55	61	987		
544	Jersey	6/0/51	2/7/55	48	947		
158	Jersey	12/2/53	Bred 3/2/55 Calved 11/26/55	24	526	835	Identical twins[a]
159 Free-martin	Jersey Guernsey	12/2/53	7/1/55	24	539	766	

[a] By external indications these animals were diagnosed to be monozygotic twins; however, the rate of release of thyroidal I^{131} in two trials during pregnancy and experimental treatment and in one trial during lactation differed considerably. No. 158 in each trial had a more rapid rate of release than did No. 159. This is taken to indicate that No. 158 had higher thyroid gland activity. This difference may be responsible in part for the higher lactational response of No. 158. On the basis of these differences, it is possible that these twins are dizygotic.

milk yield was reached. Twice daily milking at about 7 A.M. and 4 P.M. was commenced on the day after the first injection. The mammary secretion at first consisted of a sticky honey-like material, which rapidly developed a colostral appearance, gradually changing to normal-appearing milk. A marked increase in milk yields commenced between the third and sixth days in the Jersey heifers.

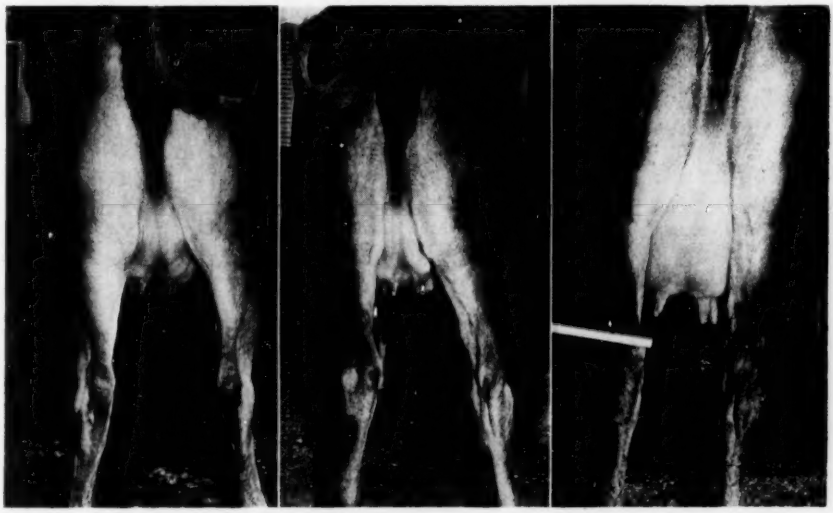

FIG. 1. Views of udder of Jersey heifer No. 580. Center — before experiment was begun. Left — at end of 180 day injection period. Right — after lactation was initiated by means of estradiol benzoate.

In these animals the injection of estrogen was stopped after 14 days. Since in the Holstein heifer (No. 402) copious milk production was not started until the 15th day, estrogen injections were continued for seven additional days.

The Guernsey freemartin was the only animal which failed to come into milk. Even though estrogen injections were continued for 26 days, she never yielded more than about 85 ml. of milk per day.

The rapid rise in milk secretion in each animal is shown in Figures 2 to 4. The time required to reach maximum daily milk production (based on a weekly average) varied from 5 to 14 weeks (Table 2). The rising segments of the lactation curves were somewhat more prolonged than in cows that calve normally. This was probably due to the fact that milk removal was commenced at the time the lactation phase was stimulated. A comparable rise in milk secretion in normal pregnancy in heifers milked for 40 days prior to parturition has been observed *(22)*. It is probable that if the heifers had been allowed to "bag up," as is usual in normal animals, the rising segment would have been comparable.

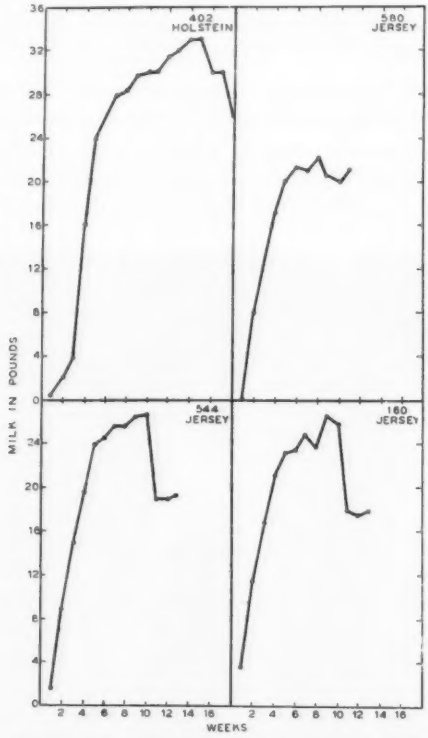

FIG. 2. The rising segment of the lactation curves experimentally induced by the daily injection of 3 mg. of estradiol benzoate for 14 days. Since No. 402 had not come into milk at the end of 14 days, injections were continued for 1 week. Note milk yield continued to rise after estrogen was stopped.

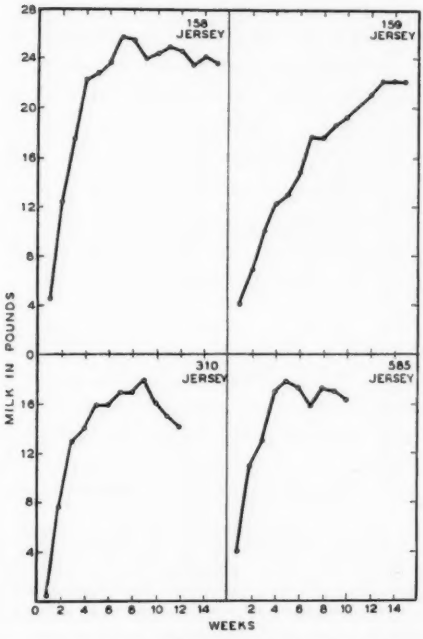

FIG. 3. The rising segment of the lactation curves of heifers induced to lactate with estrogen. Jersey No. 158 calved normally, whereas No. 159, her monozygotic twin, was induced to lactate experimentally. Her maximum milk yield was 85.2% as much as that of the normal twin.

The maximum daily milk yield and its FCM equivalent for each animal is presented in Table 2. The yield varied from 12.2 lb. of milk in cow No. 583 with only three functional quarters to 33.3 lb. in No. 402.

TABLE 2
Production after the initiation of lactation

Herd No. & breed	Time to reach maximum milk prod.	Milk yield at maximum	Fat content at time of maximum production	FCM Maximum	Remarks
	(*wk.*)	(*lb/day*)	(*%*)	(*lb/day*)	
310—Jersey	9	18.2	6.4	25.3	
580—Jersey	8	22.9	5.1	27.2	
583—Jersey	9	12.2	5.1	13.9	One blind teat
585—Jersey	5	18.2	5.7	22.3	
402—Holstein	14	33.3	3.7	31.3	
544—Jersey	10	29.6	3.5	27.4	
158—Jersey	7	26.3	4.4	27.9	
159—Jersey	12	22.4	3.8	21.7	
160—Jersey	8	27.3	4.6	29.8	
Freemartin Guernsey	—	Lactation in quantity not initiated			

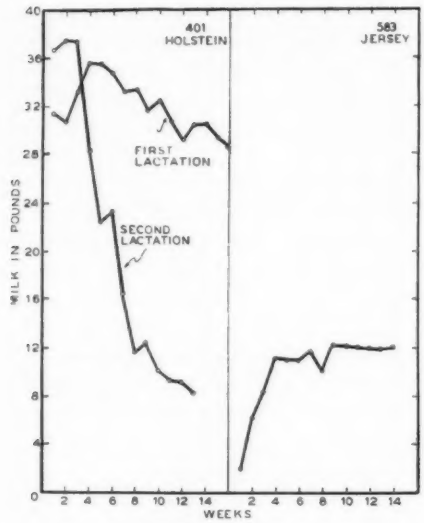

FIG. 4. The first and second lactation records of Holstein No. 401 after normal pregnancy in the University herd. She was a dizygotic twin of No. 402, which was induced to lactate experimentally (See Fig. 2). The maximum milk production of No. 402 was 93.0% as great as that of No. 401 in her first lactation. No. 583, with three functional quarters, had the lowest maximum milk yield under experimental conditions.

For studies such as this the ideal comparison would be to use identical twins with similar productive capacity and under similar managerial treatment. Only one such comparison is possible, namely, in cows No. 158 and 159. One heifer calved normally (No. 158) and produced a maximum of 27.4 lb. of milk at the maximum. Her twin (No. 159) was stimulated experimentally and produced a maximum of 22.4 lb. of milk, or 85.2% as much (Table 3).

TABLE 3

Comparison of maximum milk yield of the experimental animals with related animals

Herd No.	Maximum daily milk production of related animals		Maximum daily milk production of experimental animals	Comparison
	(No. of animals)	*(lb/day)*	*(lb/day)*	*(%)*
310	17	27.6	18.2	65.9
580	4	16.7	22.9	137.1
583	4	16.7	12.2	73.1
585	2	14.3	18.2	127.3
402	1	35.8 (Twin 401)	33.3	93.0
160			27.3	
544			29.6	
159	1	26.3 (Identical twin to 158)[a]	22.4	85.2

[a] See footnote to Table 1.

In cows No. 310, 580, 583, and 585, Jerseys from the University herd, data for the average maximum daily production of paternal and maternal half-sisters were available. Their production in comparison with the production of the experimental animals varied from 65.9% to 137.1%.

Holstein heifer No. 402 was the twin of No. 401, a fertile animal, which had two lactation records in the University herd (Figure 4). Under experimental conditions No. 402 produced 93.0% as much milk as did her dizygotic twin.

Production records are not available on relatives of Jersey cows No. 160 and 544. Their records of maximum production were thought by their owner to be approximately equal to the production which he would have expected had they calved normally.

On the basis of all available comparisons, it is concluded that the experimental stimulation of the growth of the udders and the initiation of milk secretion in these heifers rather closely approached the production which might have been obtained had they calved normally. Since apparently none of these animals had an inheritance for high production, it is not known whether one would have been equally successful with high producing animals.

The failure to stimulate appreciable quantities of milk in the Guernsey freemartin cannot be explained at this time. Whether better success would have been obtained had she been prestimulated with estrogenic hormone for a period to obtain a more extensive duct system before attempting to grow the lobule-alveolar system is not known. Additional freemartin heifers are now available to determine, if possible, the cause of the failure in this animal.

Discussion. Greater problems are involved in evaluating the results of the experimental induction of mammary gland growth in cattle than with small experimental animals. In the latter, gross whole mounts of the glands can be prepared for visual examination. The determination of the nucleic acids (DNA and RNA) of the gland indicates the increase in the number of cells *(6)*. The methods developed by Mixner and Turner *(12)* and Richardson *(17)* for examination of the goat's udder require sacrifice or surgical removal of a half and then only the type of growth and not more than a rough approximation of the extent of growth can be estimated.

A lactation index of mammary gland growth (the present experiments) raises the question as to the completeness of the stimulation of lactation in the cells by the estrogen.

The requirement of progesterone in the experimental growth of the udder has been questioned by some investigators because of the fact that in a few animals estrogen alone has been able to stimulate considerable lactation. On the other hand, the response to estrogen has been very variable, and in many animals very little milk has been obtained. The lactation induced by estrogen is believed by the writers to be due primarily to the amount of gland tissue present at the time the estrogen is administered (the lactation stimulating effect) rather than to a stimulation of lobule-alveolar growth.

It is the firm conviction of the writers that the complete growth of the lobule-alveolar system of the cow requires progesterone just as it is required in the growth of experimental animals. It is possible that considerable amounts of progesterone may be secreted by the adrenal under certain types of stress, which may contribute to the requirement of this hormone. Research on the best synergistic ratio of estrogen and progesterone in experimental animals indicates that the ratio in most animals is of an order of 1:1000 or more. The early determination of a narrow ratio in the rabbit, which is believed to be an exception owing to the low progesterone requirements *(28)*, has led previous investigators in the field to use narrow ratios of the two hormones in work with goats and cattle. As a result, the large amounts of estrogen used have over-ridden the physiological effect of the progesterone and induced precocious lactation rather than mammary gland growth *(3)*.

The ratio of the two hormones used in the present experiments seems to be based on sound physiological observations in regard to the requirements of progesterone for the maintenance of pregnancy in heifers and on the low requirements of estrogen for physiological effects.

The use of a 180-day injection period was based upon the time required for lobule-alveolar growth in experimental animals and upon observation in cattle. Since the growth of the glands appears to be rapid at first and slows down toward the end of the period, in practice a shorter period of injection might eventually be used to attain a close approach to complete growth.

Practical use of a method of inducing growth and lactation in sterile heifers will be dependent upon the development of methods of oral administration of the two ovarian hormones. Synthetic estrogens have been shown to be effectively administered orally. In experiments now in progress (to be reported later) the feeding of 10 mg. daily of diethylstilbestrol and the injection of 100 mg. of progesterone daily have been found satisfactory. Oral administration of compounds with progesterone-like biological activity has been described. These or related compounds may soon become available for use in this work.

Once the growth of the lobule-alveolar system has been attained, the second problem is to induce the intense secretion of lactogenic and other hormones to initiate and maintain the secretion of milk. It has been suggested *(10)* that lactation before and after parturition is induced by the over-riding physiological effect of estrogen in relation to progesterone. Estrogen stimulates the secretion of the lactogenic hormone, which, acting upon the gland cells, initiates and maintains milk synthesis. In late pregnancy, the udder swells with the accumulating milk. In our experiments, the udders showed no swelling until estrogen was administered at the close of the growth phase (Figure 1). Then swelling of the udder was comparable to that occurring in late pregnancy.

The best bethod of initiating the lactation process is not known. The present method involves the injection of 3 mg. of estradiol benzoate daily for 14 days or more. Lactation was initiated by this process and milk yields continued to increase for a number of weeks after the estrogen injections were stopped (see

Figures 2-4). Whether higher maximum yields of milk could be obtained by gradually increasing the estrogen injections for a longer period must await further study. It would be more like the normal process in late pregnancy.

One of the greatest problems in this field of experimentation is the variation in the production of milk by individual heifers. How does the maximum yield of milk of the experimentally induced animals compare with what they would have yielded had they calved normally? Identical twin heifers are the ideal experimental animals. In other heifers, only gross comparisons with the production of related animals is possible. On the basis of these various comparisons, it is believed that the present methods of the induction of lactation approached that which might have been expected of these animals had they calved normally. It is believed that the basic plan is sound, but modifications in the procedure may make possible the stimulation of milk production equal to or in excess of the normal rate.

In a number of the so-called "sterile" heifers used in this experiment it has been possible to obtain conception during their experimental lactation period. As these animals calve, it will be possible to compare their normal and experimental production. In the animals which cannot be bred, a second course of hormone injections will be administered to determine whether lactation can be reinduced after a dry period.

The composition of the milk secreted by the heifers induced to lactate was not determined except for its fat content, since an extensive study of the milk constituents of normal and experimentally induced milk of identical twins has been conducted in New Zealand *(13)*. It was reported that the colostral phase lasted longer in the experimental animals than after normal parturition. For most of the sets of twins, the fat and protein contents of the milk of the treated animals were higher than in the corresponding samples from the twins in normal lactation. In all cases the values obtained were within the range for normal chemical composition. In mineral constituents, the values in the twins were very similar. Since the experimental process of lactation induction is believed to stimulate the hormones effective during normal pregnancy and parturition, there is every reason to expect milk of normal composition to be secreted.

SUMMARY

An experiment is reported concerning the experimental induction of growth of the mammary glands of the cow's udder followed by the experimental induction of milk secretion.

The group of heifers included six Jerseys and one Holstein that had failed to conceive, one set of Jersey twins diagnosed as monozygotic, and one freemartin Guernsey.

To induce lobule-alveolar mammary gland growth in these animals, they were injected daily with a mixture of 100 mg. of progesterone and 100 γ of estradiol benzoate for a period of 180 days. During this period there were few external signs of change of the udder except slight lengthening of the teats and

hyperemia of the skin. Estrous cycles were inhibited, and mounting and pelvic changes observed when estrogen alone is injected were not observed.

To induce milk secretion, 3 mg. of estradiol benzoate were injected daily for a period of 14 days or more. A marked increase in milk yield commenced from the third to sixth days in the Jerseys but was delayed in the Holstein. The Guernsey freemartin failed to come into copious lactation. Maximum milk yields were reached 5 to 14 weeks after the beginning of stimulation.

On the basis of various comparisons, it was concluded that the maximum production of milk rather closely approached the milk production which might have been obtained had they calved normally.

The failure of the Guernsey freemartin to respond to the treatment cannot be explained at this time.

Although complete chemical analysis of the milk secreted by these heifers was not made, evidence for the normality of milk composition furnished by pairs of identical twins under similar experimental treatment indicates that the milk stimulated by this method would be normal.

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THE RELATIVE IMPORTANCE OF ATMOSPHERIC HUMIDITY IN INFLUENCING THE GRAZING PERFORMANCE OF DAIRY COWS DURING WARM WEATHER

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Six lactating cows were studied under a continuous pasture treatment from the latter part of June until the latter part of August to determine the relative importance of atmospheric vapor pressure to daytime grazing habits of the animals. An insignificant relationship was found between variations in grazing and changes in relative humidity. *Editor.*

In the study of the heat tolerance of dairy cows under natural conditions much of the work has been done without partitioning the weather into its component elements to determine their separate effects. Consequently, the effect of humidity on cow comfort and behavior has been to a large extent a moot question among cattlemen.

Owing to the use of relative humidity instead of vapor pressure, or absolute humidity, as a measure of the water vapor of the atmosphere, some of the research regarding the effects of humidity on an animal under natural conditions has been confused. In the early work of Seath and Miller *(10)* high humidity, when expressed as relative humidity, was shown to be statistically unimportant as a factor influencing cows' summer comfort, but as temperature was not held constant another variable, vapor pressure, was included. Lee *(7)* states that "for animal thermo-physiology, it is the absolute humidity, or rather the associated vapor pressure, which matters." Bedford *(1)* and Dordick *(3)* take a similar stand. Johnson and Frye *(5)* and Riek and Lee *(9)*, working in controlled atmospheres, have shown that absolute humidity does have an important effect on cow comfort. Though workers in Missouri *(8)* use relative humidity in their reports on environmental physiology, they also give the dry bulb readings, thus making it possible to translate the data to any index desired.

During the summer months in the lower South, time spent by dairy cows grazing between morning and evening milkings may range from less than 10% to more than 65%. The object of this study was to determine the relative importance of humidity in causing the variations in the grazing performance of dairy cows during summer weather in southern Louisiana.

EXPERIMENTAL PROCEDURE

Six lactating cows, three Holsteins and three Jerseys, were studied under a continuous pasture treatment from the latter part of June to the latter part of

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August. In addition to the pasture herbage, which was fairly good throughout the study, the regular herd concentrate was fed according to production. The cows went to the pasture at 7:00 A.M. and returned to the milking barn at 2:30 P.M. each day during the time under observation. Grazing performance was recorded on 16 days during the season.

The days chosen for this study were similar in air movement, per cent clear sky, and lack of precipitation. To prevent a weather lag, no day was chosen unless the preceding day was similar. Wind velocity was low and varied little, which is typical of this region during summer *(4)*. Although no instrument was used to measure air velocity, observations were made and recorded. Except for an occasional gust of wind, air movement was very mild to mild. Checking with the local weather bureau revealed that such breezes would average below 5 m.p.h. Workers at the Missouri Experiment Station *(2)* state that European-evolved cows adjusted themselves to changes in air movement through environmental temperatures ranging from 18° to 80° F. At 95° milk production and feed consumption were lower at low air velocity (0.4 to 0.5 m.p.h.) than at high velocity (7 to 10 m.p.h.), but this depression may have been within the limits of experimental errors. Other work in Missouri *(6)* showed that cows were benefited by air velocities of 8 to 9 m.p.h. at an environmental temperature of 95° F. Only temperature and humidity remained as important environmental variables. Wet and dry bulb temperatures were recorded at 2:30 P.M. each day, and body temperatures were taken as soon as the cows entered the barn (at approximately 3:00 P.M.).

As the days selected did not follow in consecutive order, milk yields were not reported. Correlations were computed according to Snedecor *(11)*.

TABLE 1

Means and standard deviations and range of grazing time and body temperature of cows and weather data

Cow	Grazing time as per cent of total time in pasture			Body temperature		
	Mean	Standard deviation	Range	Mean	Standard deviation	Range
1	32.1	± 12.07	16–62	103.5	± 0.66	102.4–104.5
2	28.6	± 12.41	11–65	102.6	± 0.43	102.0–103.2
3	24.3	± 12.21	7–48	103.5	± 0.58	102.4–104.3
4	33.4	± 11.37	16–57	103.5	± 0.54	102.7–104.6
5	28.4	± 10.40	10–49	102.7	± 0.49	102.0–103.6
6	25.8	± 9.63	11–47	103.8	± 0.64	102.7–104.8
Average	28.8	± 11.34	12–55	103.3	± 0.57	102.4–104.2

Weather								
Dry bulb			Vapor pressure			Relative humidity		
Mean	Standard deviation	Range	Mean	Standard deviation	Range	Mean	Standard deviation	Range
(°F.)			*(mm.Hg.)*			*(%)*		
90.4	± 1.89	87–94	18.6	± 1.84	15.6–21.3	50.6	± 3.62	42–56

TABLE 2

Correlation coefficients among environmental factors, body temperature, and per cent time spent grazing

Variable	r values				
	X_1	X_2	X_3	X_4	X_5
X_1 — Grazing	—	–0.665	–0.106	–0.585	—
X_2 — Vapor pressure	—	—	0.143	0.723**	0.540
X_3 — Relative humidity	—	—	—	0.079	—
X_4 — Atmospheric temperature	—	—	—	—	0.320
X_5 — Body temperature	—	—	—	—	—
Degrees of freedom, 94					
Partial correlations					
	$r\,12:4 = -0.430$**				
	$r\,14:2 = -0.019$			$r\,25:4 = 0.746$**	
	$r\,13:4 = 0.189$			$r\,45:2 = 0.021$	
Degrees of freedom, 93					

** Significant at 1% probability

RESULTS AND DISCUSSION

The means and standard deviations of means and correlation coefficients for all data are shown in Tables 1 and 2, respectively.

Grazing and atmospheric temperature. Because of the high correlation, 0.723, between temperature and vapor pressure, the similarity of the simple r values, –0.585 and –0.665, between grazing performance and temperature and between grazing performance and vapor pressure, respectively, is not surprising. But when analysis of partial correlation was used, the correlation between grazing and temperature, independent of vapor pressure, had a coefficient of only –0.019, which is not significant. Variations in air temperature also showed very little relationship to variations in body temperature, the partial correlation coefficient being –0.021. As a cow's behavior is closely related to her comfort, this small correlation was not unexpected. Research by Louisiana workers *(5)* in controlled atomspheres showed that high air temperatures without high humidity were only slightly effective in causing rising body temperatures.

Grazing and vapor pressure. The range of vapor pressure was from 15.6 to 21.3 mm. Hg. The simple r value, –0.665, for time spent grazing and vapor pressure was but little larger than the value, –0.585, for grazing and air temperature, but when the effect of air temperature was eliminated by analysis of partial correlation the coefficient for vapor pressure and time spent grazing was –0.430, which is highly significant. This indicated that pressure was probably responsible for most of the simple r value between air temperature and time spent grazing. Vapor pressure had a high correlation, 0.746, with body temperature also. This could be expected as a cow's activity is known to decrease under heat stress, and the variation among cows in time spent grazing would indicate considerable difference among the cows in heat tolerance. Thus, the separate effect of vapor pressure was shown to have a relatively important bearing on the activity and comfort of dairy cows in summer weather. Because of the low correlation between

vapor pressure and relative humidity under field conditions they could not be used interchangeably in this study.

Grazing and relative humidity. The relationship between variations in grazing and changes in relative humidity was insignificant, the partial correlation coefficient having a value of 0.189. This small positive value is understandable when the small relationship of relative humidity to vapor pressure and temperature is noted.

SUMMARY AND CONCLUSIONS

Six lactating cows, three Holsteins and three Jerseys, were studied under a summer pasture environment in southern Louisiana to determine the relative importance of atmospheric vapor pressure to the daytime grazing of dairy cows.

On 16 days representative of Louisiana summer weather the data were collected. These days, which were very similar, were mostly clear, with typically mild summer breezes and no rainfall. This left only temperature and humidity as factors that might be importantly related to daily variations in the grazing behavior of the cows.

The correlation coefficients among the different factors are as follows: grazing and vapor pressure independent of atmospheric temperature –0.430 ($P < 0.01$); grazing and atmospheric temperature, independent of vapor pressure, –0.019 ($P > 0.05$); grazing and relative humidity independent of atmospheric temperature 0.189 ($P > 0.05$); vapor pressure and atmospheric temperature 0.723 ($P < 0.01$); vapor pressure and relative humidity 0.143 ($P > 0.05$); body temperature and vapor pressure independent of atmospheric temperature 0.746 ($P < 0.01$); body temperature and atmospheric temperature independent of vapor pressure –0.021 ($P > 0.05$).

This study revealed the following:

1. Variations among days in atmospheric vapor pressure showed a highly significant correlation with daily variations in daytime grazing and body temperature of dairy cows.

2. Variations among days in the range of atmospheric temperatures used in this study appeared to be unimportant as an influence on grazing performance and body temperature.

3. Relative humidity was not significantly correlated to vapor pressure or grazing performance.

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SOME OBSERVATIONS ON THE BEHAVIOR OF GRAZING LACTATING COWS [1, 2]

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By using four Holstein cows in each of two trials running for 10 consecutive days, data were obtained on the manner in which grazing lactating animals allocate their time. In general, the animals exhibited four main grazing periods daily. Least grazing was done between 8:00 and 11:00 at night. *Editor.*

The study of animal grazing behavior is not new. In 1797, Anderson *(1)* observed the grazing habits of cattle and as a result of his observations recommended that a system of rotational grazing be followed in order to utilize pastures most effectively. Much interest has recently been shown in the behavior of the grazing animal as evidenced by the extensive reviews by Tribe *(13)* and Hancock *(6)*.

This investigation was conducted to obtain data on the manner in which grazing lactating animals allocate their time to the various activities in which they are daily engaged and to study some of the variations in these activities which are brought about as a result of certain environmental conditions. Since Harker *et al. (9)* in Africa have shown that grazing behavior studies must be considered in relation to the particular environmental conditions under which they are conducted and since relatively few such studies have been made in the southeastern part of the United States, these observations should be of interest to workers in this particular area.

EXPERIMENTAL PROCEDURE

The studies reported herein were conducted in two trials with eight Holstein cows. In each trial four cows were under continuous observation for 10 consecutive days. A 6-acre area subdivided into six 1-acre plots was rotationally grazed by each group of four cows. One-third of the area was seeded to a mixture of ladino clover and orchard grass, one-third to ladino clover and Kentucky 31

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[1] Contribution from the Department of Dairy Science.

[2] Data presented are based on a thesis submitted by H. L. Fisher to the Graduate Faculty of the Virginia Polytechnic Institute in partial fulfillment of the requirement for a Master of Science degree.

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fescue, and one-third to alfalfa and orchard grass. The pasture mixtures were the same in each trial. In each trial a portion of the pasture area grazed had been renovated the year previous; the remainder was non-renovated. In addition, the area grazed during Trial 2 was irrigated by means of a portable sprinkler system when 50 to 65% of the available water in the root zone was depleted.[4] Half of the cows in each trial received a simple grain mixture at the rate of 1 lb. of grain for each 6 lb. of milk produced in addition to the pasture herbage. The cows were milked twice daily in a barn located about 0.3 to 0.5 of a mile from the pasture plots. Thus the cows were off the pasture for about 1 hour and 45 minutes in the morning and about 2 hours in the afternoon.

The following activities were recorded to the nearest minute for each animal in each trial: standing time (total time spent in standing position); lying time (total time animals were reclining whether ruminating or not); grazing time (entire time spent selecting and eating herbage); loafing time (time spent standing or being milked while not grazing and including that portion of the ruminating time spent in the standing position); rumination time (including the total time, both standing and lying, spent in regurgitation, reinsalivation, remastication and reswallowing, and the short intervals between boluses); number of drinks, urinations, and defecations. Other activities, such as the number of bites per minute while grazing and the number of jaw movements per minute while ruminating were also recorded at various intervals. A continuous record of the air temperature and relative humidity was made throughout the study.

RESULTS

The results of the two trials are presented in Table 1.

Standing and lying time. The average standing time of the eight cows for the two 10-day observation periods was 900 minutes per day, with times for individual cows ranging from 805 to 1,010 minutes. Thus the average lying time was 540 minutes per day since the standing and lying times for each day necessarily equaled 1,440 minutes. There was a difference of only 15 minutes in the average standing time per cow per day between the first and second observation period. A large portion of the lying time was spent during the hours of darkness; however, a major period of lying occurred during the day immediately following the first grazing period of the day after the morning milking.

Grazing time. The average time spent grazing was 542 minutes per day with a range of 481 to 643 minutes for individual cows. In other words, about 38% of the total time of the animals was spent grazing. The difference in the mean grazing time between the first and second observation period was small, being about ½ hour.

The cows differed significantly in the amount of time spent grazing in both periods. Also, the variation in grazing time due to days was significant in each period.

[4] The Department of Agricultural Engineering was responsible for irrigation of the pasture plots.

TABLE 1

Summary of observations made during the first and second trial together with daily milk production and body weight of the cows

	Trial 1						Trial 2					
	Animals				Mean	As % of 24 hr.	Animals				Mean	As % of 24 hr.
	1	2	3	4			5	6	7	8		
Time spent *(minutes)*												
Standing	942	851	1010	826	907	63	857	978	805	817	892	62
Lying	498	589	430	514	533	37	583	462	635	623	548	38
Grazing	582	522	643	483	558	39	562	481	517	544	526	37
Loafing	360	330	367	343	350	24	296	611	288	272	367	25
Ruminating	495	501	491	480	494	34	514	554	514	587	542	38
Number of:												
Defecations	16.4	11.5	14.7	15.3	14.5		15.9	16.0	12.6	17.3	15.4	
Urinations	8.5	8.9	10.3	10.9	9.6		6.7	6.1	10.4	14.0	9.3	
Drinks	4.8	4.6	6.2	3.8	4.8		3.0	2.1	2.5	4.6	3.0	
% of grazing time between A.M. and P.M. milkings	48	47	43	45	46		53	50	56	53	53	
% of grazing time between P.M. and A.M. milkings	52	53	57	55	54		47	50	44	47	47	
4% FCM *(lb. per day)*	24.6	22.3	50.5	50.1	36.9		40.8	21.9	26.7	34.1	30.9	
Body weight	1,209	1,090	1,062	1,318	1,170		1,166	1,297	1,090	1,198	1,188	
Daily temperature *(° F.)*[a]					76						64	
Relative humidity (%)[a]					45						44	

[a] Taken in shade.

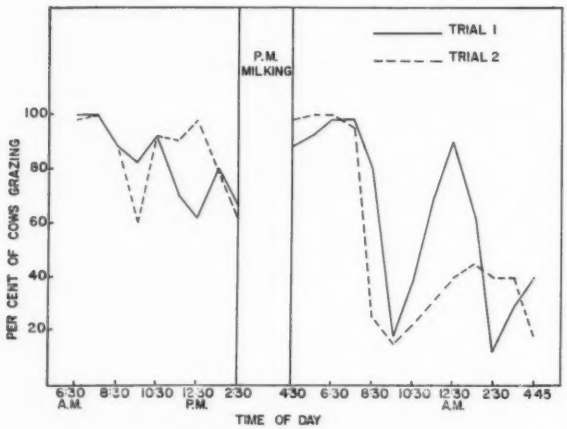

FIG. 1. Distribution of grazing time over 24-hour period

In general the animals exhibited four main grazing periods daily. This is shown graphically in Figure 1. They tended to graze more intensively and for longer periods of time immediately on reaching the pasture after the morning and evening milkings. This was especially true after the evening milking. In addition, each group of cows exhibited a grazing period of 1 to 2 hours before noon and another period of similar length around midnight. The cows preferred to graze during the hours of daylight, and it is interesting to note that in each trial the least amount of grazing was done between the hours of 8:00 and 11:00 at night.

If day grazing and night grazing are defined as those periods of time the animals spent in grazing between the morning and evening milkings and between the evening and morning milkings, respectively, then it can be seen from Table 1 that approximately 50% of the grazing was done during the day and 50% at night. However, the day:night ratio for Trial 1 was almost directly reversed to that observed during Trial 2 (46:54; 53:47). This was thought to be due to the difference in mean daily temperature between Trials 1 and 2. In this connection it is interesting to note that a significant negative correlation between mean daily grazing time and mean daily temperature was found for Trial 1 but not for Trial 2. The correlations observed between grazing time and milk yield or body weight were nonsignificant.

Loafing time. Loafing time accounted for 358 minutes of the cows' day (mean for both trials) or about 25% of the total time. Since approximately 60% of the loafing time was "forced" (due to milking routine) it had no particular significance in itself. Although the mean loafing time for Trial 2 was higher than for Trial 1, this resulted from the much longer loafing time of Cow 6. The explanation for this increased loafing time is not obvious; however, this animal grazed for a shorter period of time than any of the other animals observed during Trial 2.

Ruminating time. The average time spent ruminating per day was 518 minutes. Rumination occupied 34% of the cows' time during Trial 1 and 38% during Trial 2. The difference in ruminating time between days was highly significant for both periods. In Trial 2, cows were also observed to differ significantly in ruminating time.

In general, rumination occurred during four or five major periods throughout the day. The periods of rumination varied from 31 to 51 minutes and averaged 42.9 minutes for both trials. A major portion of the ruminating was done during the hours of darkness and in the lying position. Rumination rarely occurred while walking,, although in several instances some cows were observed to do so, especially while going to and from the milking barn.

Number of drinks. The cows averaged 3.9 drinks per cow per day. The frequency of drinking was higher during Trial 1 than Trial 2 (4.8 vs 3.0). The increased water consumption during Trial 1 was apparently due to higher environmental temperatures during this period and the intake of forage which was higher in dry matter content than that encountered during Trial 2. The cows had access to water only when they were in the pasture lots, and they usually drank immediately upon reaching the pasture following the morning and evening milkings. Very few drinks were taken during the hours of darkness.

Defecations and urinations. Feces were voided an average of 15.0 times and urine 9.4 times per 24-hour period. Defecation or urination or both invariably occurred immediately after getting up if the animal had been lying down for an appreciable length of time. The distribution of defecations and urinations is presented in Table 2.

TABLE 2

Distribution of defecations and urinations over 24-hour period (% of total number)

Period	Trial 1		Trial 2	
	Defecations	Urinations	Defecations	Urinations
During A.M. milking and en route to and from pasture	11.0	6.2	11.8	8.8
On pasture between A.M. and P.M. milkings	34.2	33.0	34.6	37.4
During P.M. milking and en route to and from pasture	10.3	13.4	14.4	11.0
On pasture between P.M. and A.M. milkings	44.5	47.4	39.2	42.8

Miscellaneous observations. More than 500 counts were made at various intervals during both trials of the number of bites per minute while grazing and the number of jaw movements per minute while ruminating. The results showed that the animals averaged 58 bites per minute while grazing and 64 jaw movements per minute while ruminating. Jaw movements per minute during rumination were quite constant. On the other hand, bites per minute while grazing appeared to be influenced by the sward, the rate being high when young rapidly

growing herbage was grazed and low when herbage at an advanced stage of maturity was available.

The feeding of 1 lb. of grain for each 6 lb. of milk resulted in a decrease in grazing time of about 7½%.

DISCUSSION

The observations reported herein are in general agreement with those made by several other investigators *(2, 5, 11)*.

The results of this study as well as others *(2, 5)* demonstrate that there is a natural tendency for the dairy cow to divide her grazing time about equally between the A.M.-P.M. and P.M.-A.M. milkings. From the standpoint of practical animal management this fact is of greater importance than the total hours of daylight and darkness devoted to grazing. Thus, as pointed out by Castle *et al. (2)*, it appears advisable for the dairyman to provide day and night pastures of approximately equal size and with herbage of similar quality else intake will be reduced or practically all of the daylight hours will be devoted to securing feed.

All results to date indicate that animals prefer to graze during the daylight hours and it is only when conditions are unfavorable that an appreciable amount of time is spent grazing during darkness. However, the assumption made by Cory *(3)* that once animals "bed down" at night they remain down until daylight is not valid in the light of the present study and those of Hancock *(5)*.

The drinking habits of the animals in this study indicate that if dairy cows have access to water before being placed on the night pasture, no adverse effects should result because of lack of water in the pasture area.

Several workers have indicated the practical usefulness of grazing observation studies. Thus, Levy *(12)* suggested that a knowledge of the grazing habits of cattle may help eradicate feed flavors in milk. Hughes and Harker *(10)* have shown how behavior studies may be used to increase the accuracy of weighing animals on pasture, and workers in this country *(4)* have stressed the fact that information regarding the grazing habits of sheep aids greatly in the proper use of range pastures. On the other hand, continuous and meticulous observations of the grazing animal did not reveal the reasons for the day to day fluctuations in the incidence of bloat, and the condition could not be prevented by changing the grazing habits of the animals *(8)*.

Since it has been shown *(6, 7)* that animals adapt their grazing habits rapidly to new or adverse conditions, it would seem that grazing behavior studies can be of only supplemental value in the over-all problem of pasture evaluation. The statement by Tribe *(13)* that "care must be taken to view the data obtained by watching in the correct perspective and restraint exercised on the tendency to draw rather wide conclusions from them" seems to be worthy of the attention of those engaged in such work. Differences in the animals' endocrine and nervous systems may be factors which influence their behavior under grazing conditions. Thus, the time spent grazing, ruminating, or resting may be poorly correlated with the animal's production or size. The fact that grazing behavior is influenced by social facilitation *(14)* is still another factor limiting the value of such studies.

SUMMARY

The grazing habits and related activities of eight lactating Holstein cows have been recorded for two periods of 240 consecutive hours each. The first trial was conducted during the last part of July and the second during the first part of September. The following results were obtained.

1. The average standing time per cow was 900 minutes; lying down time, 540 minutes; and loafing time, 358 minutes per 24 hours.
2. The cows grazed an average of 542 minutes and ruminated 518 minutes per 24 hours.
3. On the average, defecations occurred 15.0 times, urinations 9.4 times, and drinking 3.9 times in 24 hours.
4. On the average, grazing time was divided approximately equally between the A.M.-P.M. and P.M.-A.M. milkings; however, high environmental temperatures were observed to increase night grazing.
5. The animals averaged 58 bites per minute while grazing and 64 jaw movements per minute while ruminating.
6. The feeding of a limited amount of grain decreased grazing time approximately 7½%.
7. The results have been discussed from the standpoint of pasture management. The contribution of such studies to the over-all problem of pasture herbage evaluation has been mentioned.

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YIELDS AND PROTEIN CONTENT OF HOLOCELLULOSE PREPARED FROM PEPSIN-TREATED FORAGES

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The effect of pepsin incubation on the yields and nitrogen content of holocellulose prepared from 10 forages ranging in protein from 3.2 to 22.0% was determined by acid chlorite treatment. A striking observation was that greater percentages of the protein than of lignin are removed by the acid chlorite treatment of forage material high in protein. *Editor.*

Retention of a considerable portion of the plant nitrogen in holocellulose preparations from forage plants has previously been reported *(1, 3, 5)*. Since many forage plants contain considerable amounts of nitrogen, particularly in the early growth stages, it may be an important contaminant of holocellulose prepared from these materials. Acid chlorite treatment (chlorine dioxide) is usually considered as primarily a delignification process and has not been proposed for the preparation of holocellulose from forages containing considerable amounts of protein.

The object of this study was to ascertain the effect of pepsin incubation on the yields and nitrogen content of holocellulose prepared from various forages by acid chlorite treatment.

EXPERIMENTAL PROCEDURE

Ten forages ranging in protein ($N \times 6.25$) content from 3.2 to 22.0% were used in this study. Nitrogen was determined by the conventional Kjeldahl procedure *(2)*. Protein was calculated on the basis that it contained 16% of nitrogen. Pepsin incubation was done at 40° C. by using 40 ml. of 1% pepsin in 0.1 *N* HCl per gram of feed, as described by Ellis *et al. (4)*. Holocellulose was prepared by the acid chlorite method of Wise *et al. (6)*. Lignin was determined by the 72% sulfuric acid method of Ellis *et al. (4)*. The theoretical holocellulose contents of the various forages were calculated as the ash, lignin, and protein-free dry matter of the extractive-free forage.

RESULTS AND DISCUSSION

Replicates of the 10 forage samples were incubated with pepsin for nitrogen determinations on the washed residues after pepsin incubation and also on the holocellulose samples prepared from the pepsin-digested forage residues. The results of this study are presented in Table 1. The protein content of the dry

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TABLE 1

Protein content of dry matter of feeds, pepsin-treated residues and holocellulose preparations

Feed	Protein in feed	Pepsin-treated residue	Retention of protein in residue	Protein in crude holocellulose		Protein in crude holocellulose[a]		Retention of feed protein in holocellulose	
				control	pepsin	control	pepsin	control	pepsin
	%								
Wheat straw	3.2	1.6	50.0	1.1	0.5	0.8	0.4	25.0	12.5
Timothy hay	6.7	2.0	29.8	4.0	0.8	1.7	0.4	25.4	6.0
Corn silage	7.4	2.4	32.4	2.1	0.3	0.9	0.2	12.2	2.7
Clover hay	10.7	4.1	38.3	4.9	1.5	3.0	0.7	28.0	6.5
Mature orchard grass	11.0	2.6	23.4	4.4	1.5	2.9	0.8	26.1	7.2
Soybean hay	11.0	2.9	26.4	4.3	1.8	2.7	0.9	22.7	8.2
Lespedeza hay	13.8	2.9	21.0	7.7	2.3	4.5	1.0	32.6	7.2
Alfalfa hay	14.5	2.8	19.3	7.6	1.6	4.1	0.6	28.3	4.1
Alfaifa silage	17.8	5.3	29.8	5.4	2.4	2.7	1.0	15.2	5.6
Young orchard grass	22.0	4.3	19.5	12.3	2.5	6.8	1.0	30.4	4.5

[a] Protein calculated as per cent of the original feed dry matter.

matter of the forages ranged from 3.2 to 22.0%. After pepsin incubation from 19.3 to 50.0% of the original protein (N × 6.25) was retained in the washed residues. Holocellulose prepared from the pepsin-digested forages contained from 0.5 to 2.4% of protein as compared to from 1.1 to 12.3% in the holocellulose prepared from control samples that had not been incubated with pepsin. Less protein was retained in holocellulose prepared from pepsin-digested forage than in holocellulose prepared from untreated forage. Only 2.7 to 12.5% of the original feed protein was retained in the holocellulose prepared from pepsin-digested forage. From 12.2 to 32.6% of the original feed protein was retained in holocellulose prepared from untreated forage.

Acid chlorite delignification treatment appears to be more effective for the removal of protein from forages than incubation with pepsin. An average of 24.6% of the original feed protein was retained in holocellulose after acid chlorite delignification, whereas an average of 29.0% of the original feed protein was retained in the washed forage residue after pepsin incubation. When holocellulose was prepared from the pepsin incubation forage residues by acid chlorite delignification, an average of only 6.5% of the original feed nitrogen was retained in the holocellulose from the 10 feeds studied. This indicates that a combination of pepsin incubation followed by acid chlorite treatment removes a large portion (av. 93.5%) of the nitrogen in the forage samples studied.

Additional studies were conducted to determine the effect of various lengths of pepsin incubation on *(a)* the protein content of the washed feed residues

TABLE 2

Protein content of four feeds, pepsin-treated residues, and holocellulose prepared after various pepsin treatments

	Alfalfa silage	Young orchard grass	Timothy hay	Wheat straw
	(%)	(%)	(%)	(%)
Protein in feed dry matter	17.8	22.0	6.7	3.2
Protein in pepsin-treated residue[a]				
pepsin — 8 hr.	5.7	6.8	2.2	3.2
pepsin — 16 hr.	5.1	5.0	1.4	1.9
pepsin — 24 hr.	5.0	4.5	1.8	1.3
Retention of feed protein (residue)				
pepsin — 8 hr.	32.0	30.7	31.9	100.0
pepsin — 16 hr.	28.6	22.5	20.9	59.6
pepsin — 24 hr.	28.0	20.2	26.4	40.4
Protein in holocellulose[a]				
untreated	3.1	7.0	2.1	0.9
pepsin — 8 hr.	1.5	2.3	0.7	0.6
pepsin — 16 hr.	1.4	1.4	0.6	0.5
pepsin — 24 hr.	1.2	1.8	0.5	0.6
Retention of feed protein (holocellulose)				
untreated	17.6	31.9	31.2	26.7
pepsin — 8 hr.	8.5	10.5	10.7	17.4
pepsin — 16 hr.	7.9	6.4	9.1	14.9
pepsin — 24 hr.	6.7	8.0	7.4	18.9

[a] Protein calculated as per cent of the original feed dry matter.

after incubation and *(b)* the protein content of holocellulose samples prepared from the pepsin-digested residues and from untreated forage samples. Four feeds were used for this study, and the results are presented in Table 2. Considerable variation occurred among the four feeds in the amount of protein remaining in the sample after pepsin incubation. There was a greater percentage of the original feed protein retained in the digested residues of the wheat straw after 8, 16, and 24 hours incubation than of the three other feeds studied. When holocellulose was prepared from the untreated feed samples more protein was retained in the holocellulose than when preparations were made from samples that had previously been incubated with pepsin for 8, 16, or 24 hours. The highest percentages of the original feed protein were retained in the holocellulose preparations from wheat straw samples after pepsin incubation for 8, 16, or 24 hours. The wheat straw dry matter had the lowest protein content of the four forage samples studied. The amount of protein removed by pepsin incubation and acid chlorite treatment is apparently not related to the amount of protein present in the original material.

Since the pepsin incubation of forage material might affect the delignification process during the subsequent acid chlorite treatment, lignin determinations were made on holocellulose samples prepared from the untreated forages and after 8, 16, and 24-hour pepsin treatments. The results are given in Table 3. Three of the forages (alfalfa silage, young orchard grass and timothy hay) showed a decrease in the lignin content of the holocellulose prepared from these forages with increasing lengths of pepsin incubation. Wheat straw holocellulose did not contain decreasing amounts of lignin when increasing lengths of pepsin incubations were given. Wheat straw holocellulose prepared with pepsin-incubated samples had higher apparent lignin contents than the holocellulose prepared from untreated samples. The retention of high percentages of the plant lignin in holocellulose prepared from young orchard grass is undoubtedly due

TABLE 3

The effect of various pepsin treatments on the lignin content of holocellulose from four feeds

	Alfalfa silage	Young orchard grass	Timothy hay	Wheat straw
	(%)	(%)	(%)	(%)
Lignin in feed dry matter	12.5	5.5	10.7	13.0
Lignin in holocellulose[a]				
untreated	3.5	3.0	1.8	1.2
pepsin — 8 hr.	3.4	3.4	1.9	1.5
pepsin — 16 hr.	3.2	2.6	1.8	1.5
pepsin — 24 hr.	2.7	2.5	1.7	1.5
Retention of feed lignin (holocellulose)				
untreated	28.0	54.5	16.7	9.2
pepsin — 8 hr.	27.2	61.8	17.8	11.5
pepsin — 16 hr.	25.6	47.3	16.8	11.5
pepsin — 24 hr.	21.6	45.5	15.9	11.5

[a] Lignin calculated as per cent of the original feed dry matter.

principally to inaccuracies of the lignin determination for certain materials. This limitation has been previously discussed *(5)*.

The results presented in Table 1 show that marked reductions occur in the protein contents of holocellulose samples prepared from materials which were pepsin-treated. This indicates that pepsin treatment would be a useful step in the preparation of low-protein holocellulose material from forages if satisfactory recoveries of holocellulose could be attained. The data in Table 4 are the

TABLE 4

The effect of various pepsin treatments on the yield of holocellulose from forages

	Alfalfa silage	Young orchard grass	Timothy hay	Wheat straw
	(%)	(%)	(%)	(%)
Theoretical holocellulose	47.7	45.8	59.8	72.1
Holocellulose yield				
untreated	49.3	45.6	59.4	73.4
pepsin — 8 hr.	39.6	41.6	58.4	70.3
pepsin — 16 hr.	39.8	40.6	57.6	69.3
pepsin — 24 hr.	38.0	38.9	54.0	67.4
Recovery of theoretical holocellulose				
untreated	103.0	100.0	99.0	102.0
pepsin — 8 hr.	83.0	91.0	98.0	98.0
pepsin — 16 hr.	83.0	89.0	96.0	96.0
pepsin — 24 hr.	80.0	85.0	90.0	93.0

results of an experiment to ascertain the recoveries of holocellulose from untreated and pepsin-treated forage samples. Lower yields of holocellulose were obtained from pepsin-treated samples than from the untreated samples in all cases studied. Recoveries of holocellulose from pepsin-treated alfalfa silage and young orchard grass samples were unsatisfactory with all of the pepsin treatments used. Satisfactory recoveries of holocellulose from timothy hay and wheat straw were obtained with an 8-hour pepsin treatment, but lower recoveries were obtained when 16- and 24-hour pepsin treatments were used. Some degradation of the holocellulose took place during the pepsin incubation period or the pepsin-treated samples were more susceptible to degradation of the carbohydrates during the subsequent acid chlorite delignification. Degradation of the holocellulose carbohydrates during pepsin incubation may be enzymatic or bacteritic. Pepsin incubation prior to acid chlorite delignification of forage samples is not satisfactory when quantitative recoveries of holocellulose are desired. This does not limit the usefulness of crude holocellulose preparations from forages since they are easily and quickly obtained and carbohydrate determinations made directly on the crude material obviate the necessity of protein and lignin analyses.

Additional studies were conducted with two high-protein forages (alfalfa silage and young orchard grass) to ascertain the effect of thymol addition during incubation with 1% pepsin in 0.1 *N* HCl and 0.1 *N* HCl without pepsin on the yields of holocellulose subsequently prepared. Replicates of each of the forages were incubated with *(a)* 1% pepsin in 0.1 *N* HCl, *(b)* 1% pepsin in

0.1 *N* HCl saturated with thymol, *(c)* 0.1 *N* HCl, and *(d)* 0.1 *N* HCl saturated with thymol. Holocellulose was prepared from each of the incubated forages and from untreated samples for determining the holocellulose yields and the protein and lignin contents. The results are presented in Table 5. The yields of holocellulose were lower when each of the various incubation treatments were used than when holocellulose was prepared from untreated samples. There was essentially no difference in the yield of holocellulose obtained after each of the incubation treatments. This indicates that the carbohydrate degradation during the incubation period may be due to acid or enzymatic hydrolysis.

Addition of thymol to the pepsin in 0.1 *N* HCl or 0.1 *N* HCl without pepsin had no marked effect on the amount of protein and lignin retained in the holocellulose prepared after these incubation treatments. Holocellulose samples prepared after pepsin incubation had much lower protein contents than the holocellulose samples prepared after incubation with 0.1 *N* HCl.

Acid chlorite treatment is generally considered as primarily a delignification process. A striking observation of the acid chlorite treatment of forage materials high in protein is that greater percentages of the protein than of the lignin are removed by the acid chlorite treatment with either incubated or untreated samples.

TABLE 5

Yields and composition of holocellulose prepared after various incubation treatments

	Alfalfa silage			Young orchard grass		
	Holocellulose yield	Protein retention	Lignin retention	Holocellulose yield	Protein retention	Lignin retention
	(%)			(%)		
Untreated	45.7	13.8	35.9	42.1	30.7	67.7
Pepsin	38.5	6.8	27.5	39.1	7.1	46.1
Pepsin and thymol	38.3	8.0	28.0	39.4	8.8	54.9
0.1 *N* HCl	38.0	14.6	31.0	39.8	27.3	72.7
0.1 *N* HCl and thymol	37.9	12.1	30.8	39.5	24.7	69.8

SUMMARY

Pepsin incubation prior to acid chlorite delignification of forages gave holocellulose preparations that were markedly lower in protein content than holocellulose prepared from untreated samples, but concomitant reductions in the recovery of holocellulose carbohydrates occurred.

Pepsin incubation of forage samples had no marked effect on the lignin content of holocellulose prepared by acid chlorite delignification.

Thymol did not reduce the degradation of holocellulose when forage samples were incubated with 0.1 *N* HCl or 1% pepsin in 0.1 *N* HCl.

Incubation of forage samples with pepsin prior to acid chlorite delignification is unsatisfactory when quantitative recoveries of holocellulose carbohydrates are desired.

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THE EFFECT OF PHENOTHIAZINE UPON THYROID FUNCTION IN DAIRY ANIMALS AS SHOWN BY RADIOIODINE [1, 2]

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Since phenothiazine is being used for the control of certain internal parasites in cattle, studies were made to determine the effect of prolonged administration upon thyroid function. At the dosage level administered, phenothiazine did not act as a goitrogenic agent. *Editor.*

Several investigators *(4-6)* demonstrated that the administration of the commercial form of phenothiazine (green) reduced the uptake of radioiodine by the thyroid in several species. Talmage *et al. (6-8)* reported that purified phenothiazine did not inhibit the uptake of radioiodine I^{131}. Since commercial phenothiazine (green) contains 0.3 to 0.4% organic iodine as an impurity, Nachimson *et al. (4)* compared the effects of phenothiazine and equal amounts of sodium iodide on thyroidal I^{131} uptake and concluded that the effect of phenothiazine could not be explained entirely by its iodine content. Wasserman *et al. (10)* also concluded that in addition to the iodide ion another factor, much less potent than iodine, was also responsible for a slight but definite depression of thyroid uptake of radioiodine.

Since phenothiazine is currently being recommended for the control of certain internal parasites by continuous feeding at the rate of 1-2 g. per day to cattle, studies were undertaken to determine the effect of prolonged administration on thyroid function at the 2-g. level.

EXPERIMENTAL PROCEDURE

Two Jersey heifers and one Guernsey ranging in weight from 600 to 1,000 lb. were allowed access for several months to iodized salt blocks containing 0.01% potassium iodide. Phenothiazine (green) at the rate of 2 g. per day was administered by capsule for 10 days. At the end of this time carrier-free radioiodine (NaI^{131}) was injected intravenously. Phenothiazine was continued at the same rate throughout the experiment.

Daily measurements of thyroidal I^{131} were started 24 hours after injection and continued for 18 days by means of a scintillation counting system and head

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holder described by Pipes *et al. (5)*. After several weeks the same three animals were again injected with I^{131}, and similar studies were made of the accumulation and release of I^{131} by the thyroid in the absence of phenothiazine. The data obtained in these experiments were calculated and expressed according to the methods and terminology of Brownell *(3)* as adapted to the dairy cow by Blincoe and Brody *(2)*.

Since the thyroid is secreting I^{131}-tagged hormone during the uptake phase and since the theoretical uptake (U) is determined by a series of measurements and subsequent extrapolation to zero time, it is a more satisfactory parameter of thyroid function than the maximum observed uptake. The k_4 value represents the rate constant for release of thyroid hormone from the gland.

EXPERIMENTAL OBSERVATIONS

Feeding phenothiazine to three dairy heifers at the rate of 2 g. per day reduced the theoretical uptake (U) of I^{131} to 13.2%, compared to the uptake of 35.6% during the control period of these animals. Thus, the uptake of I^{131} during the experimental period of phenothiazine feeding was reduced to 37% of that found during the control period. Measurement of the thyroidal-I^{131} release rate calculated as the k_4 value showed 0.0041 for the controls and 0.0033 for the phenothiazine-treated animals (Figure 1). However, the k_4' value (the net rate of release of thyroidal I^{131} uncorrected for reutilization of metabolized

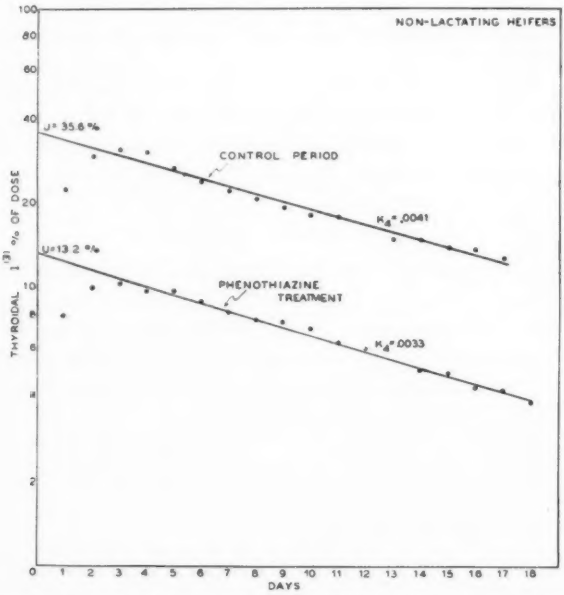

FIG. 1. Reduced rates of uptake of I^{131} and negligible changes in the rate of secretion of thyroid hormone in dairy animals under phenothiazine administration are shown.

I^{131}-tagged hormone) remained unchanged, indicating that the k_4 value was altered because of dilution of metabolized hormonal I^{131} with iodine from administered phenothiazine.

It has been shown in this laboratory by Pipes *et al. (5)* that goitrogenic agents (thiouracil and 6-methyl thiouracil) increase the rate of release twofold or more. During phenothiazine administration, however, the k_4 value was slightly reduced and the k_4' value was unchanged.

DISCUSSION

As inorganic stable I^{127} or organically bound iodine that can be metabolized to yield inorganic iodine is increased, the per cent uptake of a tracer dose of I^{131} is reduced owing to simple dilution. The present observations indicate that a similar situation exists in animals fed phenothiazine with a high iodine content.

Goitrogenic agents inhibit the synthesis of thyroid hormone and prevent the formation of organically bound iodine. However, Taurog *et al. (9)* have demonstrated that goitrogenic substances do not inhibit the uptake of iodine by the thyroid but prevent the retention of iodine. A rapid uptake of iodine was observed in these investigations, followed by an exceedingly rapid rate of release. Since the rate of release of thyroid hormone is not increased by phenothiazine, this compound evidently does not act as a goitrogen at the levels administered. These findings are in accord with those of Nachimson *et al. (4)*, who found that prolonged administration of phenothiazine did not produce a goitrogenic effect, as shown by the lack of change in thyroid size. Further evidence indicating that commercial phenothiazine is not goitrogenic has been presented by Allcroft and Salt *(1)*, who found that protein-bound iodine levels were not depressed in sheep.

This study is believed to indicate that the iodine content of commercial phenothiazine reduces the uptake of I^{131} owing to simple dilution of the tracer dose. It does not exclude the possibility that other causative agents may be present *(4, 10)*. It was shown by the technique employed that phenothiazine at the level of 2 g. per day did not behave similarly to goitrogenic agents, such as thiouracil, which increase the apparent rate of release of the thyroid hormone.

SUMMARY

Commercial phenothiazine, when administered at the rate of 2 g. per day to dairy heifers, reduced the uptake of I^{131} but did not induce hypothyroidism, as shown by the release rates of thyroid hormone from the gland. Thus at the dosage administered phenothiazine does not act as a goitrogenic agent.

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UTILIZATION OF NONPROTEIN NITROGEN IN RATIONS OF MILKING COWS UNDER HAWAIIAN CONDITIONS[1]

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Because of the relative cheapness of nonprotein-nitrogenous substances in the diet of ruminants, their use is a matter of some economic importance. Holstein cows in different stages of lactation were used in a self-feeding system which showed that efficient utilization of urea and ammonium wastes is possible. *Editor.*

The use of nonprotein nitrogen (N.P.N.) to replace part of the feed protein in the ration of dairy cattle has been shown to be acceptable *(4, 8, 11, 13, 18, 19, 20)*. Reid *(11)* has recently reviewed the literature concerning the utilization of urea by ruminants.

Carbonaceous feeds in the form of sugar cane molasses and pineapple bran are adequate in the Islands, and, because almost all of the high protein feeds are imported, a more extensive use of nonprotein-nitrogenous substances in the ration of dairy animals should be further studied.

Early work conducted at the Hawaii Station demonstrated that urea nitrogen could be utilized by steers *(10)* and growing dairy heifers *(22)*. The results with milking cows on urea rations were not as satisfactory as on non-urea rations *(20)*. However, N.P.N. has not been used extensively by dairymen in Hawaii as a protein extender.

Recent investigations at other stations *(3, 6, 11, 15, 17)* have shown that N.P.N. utilization by the ruminant is dependent on the presence of adequate soluble carbohydrate and minerals, on the amount and kind of nitrogenous compounds present in the ration, and on the different types of roughages used.

Three feeding trials were initiated to determine the usefulness of nonprotein-nitrogenous compounds in rations for dairy cows under Hawaiian conditions. The first two were designed to provide information on the feeding value of nitrogenous regenerates[2] added to pineapple bran at a level to yield a product with a crude protein equivalent of 10%. The third trial was set up to compare the feeding values of urea and nitrogenous regenerates with soybean oil meal in the maintenance of milk production.

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[1] Published with the approval of the director of the Hawaii Agricultural Experiment Station as Technical Paper No. 371.

[2] When pineapple juice is processed to recover sugar for use in preserving the canned fruit, it is passed through an ion exchange process to reduce the ash content. The ion exchange resins are regenerated with ammonium hydroxide. This exchanger waste is added to pineapple bran to provide the "ammoniated pineapple bran."

EXPERIMENTAL PLAN

Twelve-week and 15-week double reversal lactation studies as designed by Brandt *(5)* were used in the first and second trials, respectively. The third trial followed the double change-over design suggested by Cochran and associates *(7)*. The trials were divided into three equal periods of 4 or 5 weeks each. To minimize the carry-over effect of the previous ration, only data from the last 3 weeks of each period were used.

Holstein-Friesian cows in different stages of lactation with one or more previous lactations were used. The experimental period began at least 1 month after calving and ended not later than the 17th week of pregnancy. The cows in the different groups were kept in separate vegetation-free paddocks where chopped Napier grass was fed in community bunkers; thus only the average roughage consumption was measured.

The concentrate mixtures used in the experiments are shown in Table 1.

TABLE 1
Concentrate ration

Concentrate mixture	Trials I and II		Trial III		
	Control	Regenerates	Control	Regenerates	Urea
	(%)	(%)	(%)	(%)	(%)
Cane molasses	25.0	25.0	25.0	25.0	25.0
Pineapple bran	40.0	—	44.0	—	44.0
Pineapple bran + regenerates	—	47.0	—	44.0	—
Soybean oil meal, 41%	30.0	22.0	20.0	13.0	14.0
Alfalfa meal, good	—	—	7.0	13.5	11.5
Copra	3.0	4.0	—	—	—
Beef tallow	—	—	2.0	2.5	2.5
Urea	—	—	—	—	1.0
Salt	1.0	1.0	1.0	1.0	1.0
Bone meal	1.0	1.0	1.0	1.0	1.0
Total digestible crude protein	11.39	10.76	8.65	9.05	9.07
Total digestible nutrients	68.76	68.09	67.72	66.44	66.49
Cost per cwt.[a]	$ 3.48	$ 3.12	$ 2.63	$ 2.46	$ 2.56

[a] Ration costs based on feed prices current during the trials.

The large quantities of cane molasses and pineapple bran, two of the cheapest energy sources produced locally, are typical of local dairy feeds. Because of the high price of milk in relation to feed costs, the initial feed allowances were calculated on the upper limits of Morrison's feeding standards *(12)*. Napier grass was fed at a level to provide little or no left-overs in 24 hours. The additional required nutrients were supplied by the concentrates.

Complete records were maintained on milk production and feed intake. The milk yields were converted to a fat-corrected milk (FCM) by Gaines' formula *(9)*. Individual weights of cows were recorded at the beginning of the trial and at the termination of each trial period. Composite samples of all rations were analyzed under the methods described by the A.O.A.C. *(2)*. Coefficients of digestibility as given by Morrison *(12)* were used for all feed ingredients except Napier grass, urea, cane molasses, and pineapple bran. The coefficients

determined by Work *(21)* were used for these local feedstuffs. Beef tallow was estimated to have a TDN content of 214%.

Trials I and II. Comparison of ammoniated pineapple bran with nontreated pineapple bran. In the second trial, the preliminary or adjustment period was lengthened from 1 to 2 weeks. Otherwise, Trials I and II were identical. An average of 60 lb. of Napier soilage was fed per head daily. The concentrates were fed at milking time. Weekly adjustments in feed were made according to Willett's modification of Lucas's "equalized" feeding schedule for lactation *(20)*. The ration containing ion exchange plant regenerates was eaten readily in the first trial but with occasional reluctance in Trial II.

At the end of Trial II, FCM production was only 73% of the production during the week preceding the start of the experiment, compared with a milk persistency of over 90% for the 12-week period in Trial I.

Analysis of variance by Brandt's method showed no differences in FCM production between rations in Trials I and II.

Trial III. Comparison of nitrogen from urea and ammonium wastes as partial substitute for protein N. Since the trend in FCM production and persistence was not similar for Experiments I and II and because of the difficulties experienced in getting the animals to consume adequate amounts of the ration containing the nitrogenous wastes, it was postulated that a more efficient utilization of N.P.N. might be realized by extending the period of concentrate consumption over a longer interval. This was accomplished by ad libitum feeding. This experiment was also designed to compare the feeding value of nonorganically bound nitrogen (urea as a source) with organically bound nonprotein nitrogen.

In order to secure more accurate comparisons of the effects of the rations and unbiased estimates of the experimental errors, a double change-over design was used *(7)*. The experimental periods were of 5 weeks' duration with the first 2 weeks being considered as the "transitional" period.

One of the experimental animals died from acute traumatic gastritis during the last period of Trial III. The missing data on this cow were calculated according to the method described by Snedecor *(16)*.

RESULTS AND DISCUSSION

The summary of this study is tabulated in Tables 2 and 3. The results obtained from these trials are in general agreement with other data collected at this Station. Willett and associates *(20)*, using four double change-over urea feeding trials involving 26 Holstein-Friesian cows in 76 cow-periods, reported that the dairy cow can utilize nitrogen from urea in the production of milk, but not as efficiently as the nitrogen derived from plant sources.

Tables 2 and 3 show that in the three trials reported in this paper, the production of FCM was slightly lower for the cows on N.P.N. rations than for those on the control ration. These differences, however, were not statistically significant. The abrupt change-over from one ration to the other caused some animals to go "off feed" for a few days, particularly in Trial II. Results obtained by Archibald *(1)* suggest that cows may need some time to get used to rations con-

TABLE 2
Average daily milk yields, feed consumption, nutrient intake, and cost of production in Trials I and II

Ration	Trial I		Trial II	
	21 Control	34 Regenerates	21 Control	34 Regenerates
FCM yield (*lb.*)	30.7	30.1	23.2	22.0
Concentrates consumed (*lb.*)	18.3	18.4	14.7	14.7
Napier grass consumed (*lb.*)	60.0	60.0	60.0	60.0
TDN required (*lb.*)[a]	18.4	18.2	16.0	14.4
TDN consumed (*lb.*)	20.1	20.0	17.6	17.5
Total DP required (*lb.*)	2.2	2.2	1.9	1.8
Total DP consumed (*lb.*)	2.4	2.2	2.0	1.9
Total DP equivalent consumed as NPN from concentrates (*lb.*)	—	0.37	—	0.30
Total NPN consumed from concentrates (*lb.*)	—	0.08	—	0.07
Feed cost per qt. of FCM (*dollars*)[b]	0.066	0.062	0.075	0.074

[a] 1,100 lb. average weight. Nutrient intake levels calculated on upper levels of Morrison's Standards.
[b] Feed costs: Ration 21, $3.48/cwt.; Ration 34, $3.12/cwt.; Napier grass, $0.50/cwt.

taining urea; therefore, a gradual change would give a better measure of its utilization.

Napier grass intakes for Trials I and II averaged 60 lb. per head daily. For Trial III, Napier grass intake decreased to about 25 lb. per head per day. The decrease in forage intake for Trial III was probably associated with the system of feeding. In Trials I and II concentrate feeding was done at the time of milking, and weekly adjustments were made according to the previous week's production. In Trial III the concentrate feeds were continuously available in feed bunkers. All rations were accepted favorably under the concentrate self-feeding system. The average daily consumption of Napier grass was variable. Intakes

TABLE 3
Average daily milk yields, feed consumption, nutrient intake, and cost of production in Trial III[a]

Ration	Control 51	Regenerates 52	Urea 53
FCM yield (*lb.*)	46.4	45.0	45.7
Concentrates consumed (*lb.*)	43.3	44.7	41.4
Napier grass consumed (*lb.*)	23.1	27.3	21.0
Passion fruit rind consumed (*lb.*)	1.3	1.3	1.1
TDN required (*lb.*)[b]	24.9	24.4	24.6
TDN consumed (*lb.*)	32.2	33.1	30.1
Total DP required (*lb.*)	3.1	3.0	3.1
Total DP consumed (*lb.*)	3.9	4.2	3.9
Total equivalent consumed as NPN from concentrates (*lb.*)	—	0.90	0.80
Total NPN consumed from concentrates (*lb.*)	—	0.20	0.17
Feed cost per qt. of FCM (*dollars*)[c]	0.0584	0.0594	0.0551

[a] Data corrected for cow 270 (died 11-7-55) as outlined by Snedecor, 4th ed., p. 274.
[b] 1,300 lb. average weight. Nutrient intake levels calculated on upper levels of Morrison's Standards.
[c] Feed costs: Ration 51, $2.63/cwt.; Ration 52, $2.46/cwt.; Ration 53, $2.56/cwt.; Napier, $0.50/cwt.; Passion fruit rind, $0.50/cwt.

ranged from 35 lb. in Period I to 19 lb. in Period III. The reasons for using a self-feeding system included some of the newer concepts of ruminant nutrition: *(a)* continual ruminal biosynthesis, *(b)* maximal microbial activity as a function of the presence of starter materials in the proper proportions at the right time, *(c)* palatability of rations, and *(d)* economy of production.

There were no significant differences in FCM yields between the cows receiving part of their dietary nitrogen in the form of urea or ammonium wastes and those receiving their nitrogenous needs from plant protein sources.

Under the self-feeding system the animals were able to maintain a steady level of production throughout the 15-week experimental period. Concomitant to steady production, the cows gained an average of 96 lb. in body weight during the course of the study. Under the self-feeding plan the total consumption of feed was increased. The production results indicate that this increased intake of nutrients was efficiently converted into milk. Such heavy feeding is justified under Hawaiian conditions because of the high price of fluid milk ($9.83/cwt. for 4% milk) in relation to feed costs and because of the apparent need of cows in the tropics and subtropics for greater intakes of nutrients to maintain production *(20)*.

It is interesting to note that the use of N.P.N. to replace part of the feed proteins resulted in a lower feed production cost per quart of FCM.

The apparent efficiency in the utilization of nonprotein-nitrogenous compounds for milk production in the present studies may be partially explained by the continual release of ammonia over an extended period as a result of ad libitum feeding and the presence of high levels of cane molasses in the ration. Stallcup *(17)* has shown that the release of ammonia from ammoniated molasses in the rumen of a fistulated steer was not as rapid as that from urea. A possibility of a natural "ammoniation" process is suggested under the self-feeding system.

The results of this study indicate that efficient utilization of urea and ammonium wastes by dairy cows may be possible in a self-feeding system.

SUMMARY

Three trials involving 32 Holstein-Friesian cows in 12- and 15-week periods with 76 cow-periods are described.

The dairy cow is able to utilize nonprotein nitrogen in the production of milk. The results of Experiments I and II demonstrated that there were no differences in FCM production between cows fed true feed proteins and those supplied with 21% of the total digestible protein requirement in the form of nonprotein nitrogen. Maximum average daily intake of total nonprotein nitrogen was 0.20 lb. and 0.17 lb. for ammonium salts and urea, respectively.

Large daily intakes of bulky concentrates high in molasses and low in protein and total nutrients appear to be compatible in a self-feeding system. This system had an advantage in feed economy over the conventional twice-a-day feeding in the milking barn.

ACKNOWLEDGMENT

The authors express their appreciation to George E. Felton of the Hawaiian Pineapple Company, Honolulu, for the pineapple bran which was used in this study.

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TECHNICAL NOTES

LIVABILITY OF SPERMATOZOA IN DILUTERS CONTAINING YOLK-CITRATE OR NONFAT MILK SOLIDS WITH GLYCEROL

There is some evidence that glycerol may enhance or prolong fertility of spermatozoa when in yolk-citrate or milk diluters at temperatures above freezing. Polge *(6)*, with a small number of breedings, and Holt *(3)* have presented evidence that fertility of bull spermatozoa may be higher with yolk-citrate plus glycerol than with yolk-citrate alone. More recently, McLean *(5)* reported satisfactory breeding results with bull semen in boiled milk with 10% glycerol for as long as 7 days of storage. The note presented below gives the results of a study of livability of spermatozoa in three diluters, each with varying levels of glycerol.

Bull semen was first diluted at levels to obtain 30 million spermatozoa per milliliter in each of the three following diluters: *(a)* 1 part yolk and 1 part of a 2.9% solution of sodium citrate dihydrate, *(b)* 1 part yolk, 1 part of the above sodium citrate solution, and 4 parts of a 5% glucose solution, and *(c)* 8.7 g. instant Pet nonfat dry milk solids, 100 mg. cysteine hydrochloride, and 100 ml. distilled water. This last diluter was that described by Johnson *et al. (4)*. After being cooled to 5° C. each diluent was split into five parts, and to each of these parts an equal volume of a similar diluter containing different levels of glycerol was added in five steps at 6-minute intervals. The final concentrations of glycerol were, thus, 0, 1.25, 2.50, 5.0, and 10%. All diluters also contained 500 γ dihydrostreptomycin sulphate per milliliter. After storage at 5° C. for 7 days, motilities were estimated by two persons independently and without knowledge of the treatments received by each sample. The two estimates for each sample were averaged. Samples from 15 ejaculates from 9 bulls each received all 15 treatments.

Averages for the percentages of spermatozoa showing progressive motility after storage for 7 days are given in Table 1. With the yolk-citrate diluter there was a marked decline in motility with increased concentration of glycerol. In yolk-citrate-glucose there was a less marked decline. On the other hand, in nonfat milk solids, motility improved with increased amounts of glycerol; motilities with 5% or 10% glycerol were significantly greater than with no glycerol ($P < 0.05$). The glycerol × diluter interaction was highly significant ($P < 0.001$). The average progressive motility in nonfat milk solids without glycerol was not significantly different from the average for yolk-citrate or yolk-citrate-glucose without glycerol.

TABLE 1

Progressive motility of spermatozoa after storage for 7 days in three diluters with five levels of glycerol

Level of glycerol (%)	0	1.25	2.50	5.0	10.0
Diluter					
Yolk-citrate	34.5	22.0	18.3	13.3	4.1
Yolk-citrate-glucose	31.4	33.3	25.6	26.3	21.1
Nonfat milk solids	39.6	44.0	46.1	49.3	48.9

It is also interesting to note that spermatozoa in five ejaculates from four young bulls in this experiment did not survive well in either yolk-citrate or yolk-citrate-glucose. The averages for these bulls for the percentages of spermatozoa with progressive motility after 7 days in yolk-citrate with increasing levels of glycerol were 2, 0, 0, 0, 0 and in yolk-citrate-glucose, 6, 0, 0, 0, 0, respectively. In nonfat milk solids the corresponding progressive motilities were 33, 30, 31, 41, 37.

These results suggest *(a)* that caution should be taken if one contemplates a breeding experiment with semen stored several days in yolk-citrate-glycerol, *(b)* that the diluter containing nonfat milk solids, especially with glycerol added, may be superior to yolk-citrate for storage of bull spermatozoa for several days at 5° C., and *(c)* that the diluter containing nonfat milk solids may be especially beneficial for so-called "problem bulls" when yolk-citrate is the diluent. Breedings are necessary, however, to substantiate these observations. Holt *(3)* presented evidence that glycerol was especially beneficial for problem bulls when added to yolk-citrate. One must keep in mind, also, that the efficacy of antibiotics in controlling pathogens in diluters containing glycerol may be severely diminished *(1)*.

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ERRATUM

In the paper entitled *Washed and Nonwashed Butter III*, which appeared in the October, 1956, issue, the heading of Table I was printed incorrectly. The heading for Table I, which appears on page 1360, should have read "Distribution of butters according to bacteria count" instead of "Distribution of butters according to mold and yeast count."

CALL FOR PAPERS FOR THE 1957 ANNUAL MEETING OF THE AMERICAN DAIRY SCIENCE ASSOCIATION

The 52nd annual meetng of the American Dairy Science Association will be held June 26, 27, and 28, 1957, at the Oklahoma A. and M. College, Stillwater.

Members who wish to present papers must furnish titles and abstracts not later than March 1. This deadline must be met so that the complete program may appear in the Journal for May and so that the abstracts may be printed for distribution at the annual meeting. Abstracts must not contain over 200 words. Abstracts longer than 200 words will be returned to the authors by the committee.

An original and three copies of each abstract must be prepared. The original and one copy must be mailed to the Chairman, another copy to the Vice-Chairman, and a fourth to the Secretary of the Section before which the paper will be given.

The title should clearly indicate the nature of the research reported. Complete data should be presented concerning the major results of the research. Essential but not excess statistics should make the data more understandable.

Papers presented by members in the industry and by senior members of the Association are especially welcomed. If time limitations make it necessary, some papers may have to be read by title only. No member should plan on presenting more than one paper or appearing as an author of more than two papers.

Members prefer to receive mimeographed copies of pertinent material which must be presented visually (minimum 250 copies); such material includes tables of data, graphs, and brief summaries. Material for projection on screens should be used only if the preferred method is impossible.

Names and address of officers of sections to whom titles and abstracts will be sent are:

Extension Section

Chairman: James Burke, Animal Husbandry Department, Ithaca, N. Y.

Vice-Chairman: Leo Fryman, Department of Dairy Science, University of Illinois, Urbana.

Secretary: L. A. Johnson,* Dairy Husbandry Department, University of Wisconsin, Madison.

Manufacturing Section

Chairman: H. L. Templeton, 6125 Florence Blvd., Omaha, Neb.

Vice-Chairman: F. J. Babel, Department of Dairy Husbandry, Purdue University, Lafayette, Ind.

Secretary: B. H. Webb, National Dairies Research Lab., Inc., Oakdale, Long Island, N. Y.

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* On leave from Michigan State University.

Production Section

Chairman: S. W. Mead, Department of Animal Husbandry, University of California, Davis.

Vice-Chairman: S. B. Marshall, Department of Dairy Science, University of Florida, Gainesville.

Secretary: H. L. Jacobsen, Animal Husbandry Department, Iowa State College, Ames.

OUR INDUSTRY TODAY

Brief Reviews of Current Topics

Plum Island Animal Disease Laboratory Serves Livestock Industry and Mankind

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In the days of sailing ships many vessels carried a cow to supply fresh milk. In 1843, the records indicate that a British ship, the Washington, unloaded in New York a dry cow and took on a fresh one for the next trip out. Peter Dunn, a milkman, bought the captain's milked-out bovine at a bargain price; but that bargain cow, it was discovered later, became the source of a new cattle disease in America, contagious bovine pleuropneumonia, which by 1886 had spread throughout the East and westward to Ohio, Kentucky, Illinois, and Missouri. It was not until 1892 that it was declared eradicated. Contagious pleuropneumonia still menaces many parts of the world except western Europe, the western hemisphere, and a few smaller areas where it has been eliminated.

Foreign diseases today remain a constant threat to the dairy and livestock industries of this country. The problem of keeping out these foreign intruders is a growing one. Besides contagious bovine pleuropneumonia, other exotic diseases feared are rinderpest (cattle), Rift Valley fever (cattle and sheep), Teschen disease (swine), African swine fever, epivaginitis (cattle), and fowl plague (chickens and turkeys).

Foot and Mouth Disease, Number One Problem

Foot and mouth disease, of course, is the problem most dreaded. Since 1870, nine outbreaks have occurred in the United States, the last in 1929. The outbreaks in Mexico in 1946 and in Canada in the winter of 1951 are vivid reminders that any one of these diseases of foreign origin may, when least expected, hurdle the barriers erected against them. With airplane landings almost everywhere throughout the world and with more people entering America each year from the far corners of the globe where these unwanted diseases abound, mainly eastern Europe, Asia, and Africa, the risks are great.

The new St. Lawrence Seaway within a few years will allow foreign ships to discharge their cargoes right in the heart of the nation's livestock region of the Midwest. Will they bring new livestock diseases with them?

The fight continues aggressively to protect the vast livestock industry of this country. Now is no time for complacency, all agree. More knowledge, better disease control techniques, and the implementation of that new knowledge through education are needed to meet this foreign livestock disease challenge. The Plum Island Animal Disease Laboratory, a great scientific research establishment located off the tip of Long Island, appears to fulfill this need.

Secretary Benson Dedicates Laboratory

At the request of President C. F. Huffman, the writer had the good fortune, as a representative of the American Dairy Science Association, to visit Plum Island at the time of its dedication, September 25-26. Secretary of Agriculture, Ezra Taft Benson, gave the chief address. Many scientists from this country and abroad were present to inspect the facilities and to participate in the foot and mouth disease symposium the next day. To visit this $10,000,000 establishment was a great opportunity and a great privilege.

Plum Island, 1½ miles off Orient Point, is an 800-acre tract about 1 mile wide at its widest point and 3 miles long. It is the former site of Old Fort Terry, a coast artillery post established about the time of the Spanish American War. After World War II, this post was deactivated, but later it was assigned to the Army Chemical Corps, who retained it until the U. S.

FIG. 1. Principal buildings of the USDA's new Animal Disease Laboratory at Plum Island. (USDA Photo)

FIG. 2. Security guards stand at the gate to the restricted area as employees disembark from the ferry. A bus takes them to their work stations. (USDA Photo)

Department of Agriculture took over in 1952. Construction of the laboratory started in 1954.

Great Foresight in Planning

One of the most amazing things about the Plum Island development is the tremendous amount of advanced thinking and planning that went into this project. A self-sustaining island organization unit has been established because contacts with the mainland by ferry are not always certain in stormy weather. In case of emergency, food and housing for the entire staff (now 260 employees) can be provided from storage stocks and in the dormitory accommodations left by the Army. Most employees arrive and leave by ferry, which makes two trips daily over and back from Orient. However, seven families, mostly maintenance personnel, live on the Island. Later, more are expected to make their homes there as additional family units are provided.

The Island has its own shops and crews of engineers, plumbers, carpenters, sheet metal workers, steam fitters, glass blowers, and electricians; a complete laundry; a well-equipped fire station, manned day and night; a hospital, and a cafeteria. An auxiliary electrical power unit stands ready to operate should electricity, conveyed by submarine cable from the mainland, fail. Ten fresh water wells are on the Island and, in addition, facilities for pumping ocean water are provided to supplement the water supply.

No Disease Virus to Escape

The elaborate air conditioning units, the giant incinerators, and the decontamination sewer systems are protective devices of special design to keep the viruses with which the scientists work from getting away. Nothing apparently has been overlooked when it comes to preventing these animal disease germs from escaping. Trucks and busses never leave the Island. All materials, including animal feeds, pass through double-door air locks and autoclaves sterilized by live steam or gas. No contaminated supplies ever leave the buildings and restricted areas. Double fences surround the laboratory proper, and employees enter and leave only on passes checked at the gates by guards of the safety division.

FIG. 3. Infectious virus materials are handled in special safety hoods equipped with attached rubber work gloves. (USDA Photo)

On the day of my visit, we were not required to change clothing when entering the laboratory or to take a decontaminating shower when leaving as all employees must do when the laboratory is operating. In preparation for this "Open House" all viruses had been removed and safely stored about a month before and the entire building had been thoroughly disinfected to avoid all possible risks of the visitors carry-

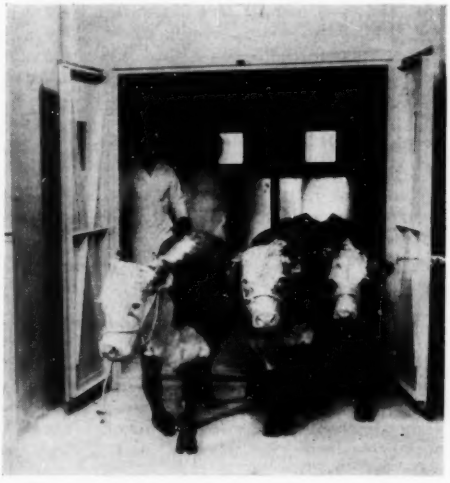

FIG. 4. Only healthy animals like those shown enter the laboratory. They are being driven out of a double door air lock room. (USDA Proto)

ing away some of the "work stock." One marvels at all the rigid precautions that are being maintained and observed at every turn.

Results Beginning to Emerge

Congress in its authorization of the construction of the Plum Island Animal Disease Laboratory directed that its staff develop a broader knowledge of how to combat contagious diseases that might be introduced into this country to threaten the livestock industry. The vesicular diseases — foot and mouth disease, vesicular stomatitis, and vesicular exanthema — were chiefly the ones in mind when Congress made its appropriation. The two latter diseases are already in the United States, but foot and mouth disease has not been diagnosed here since 1929. All three of these vesicular diseases have similar symptoms not readily differentiated in the field.

This problem of differential diagnosis is being studied. Also, foot and mouth disease is known to be caused by six different strains and types of viruses. More knowledge about these various strains is being sought. The susceptibility of the different classes of domestic livestock and wild animals to foot and mouth disease is being investigated. This is a cloven-footed animal disease. It is hoped that improved diagnostic methods will be developed, and improved immunization agents are being sought. Vaccination is recognized as having temporary value as a control measure when a foot and mouth outbreak occurs, but the established policy of the United States has been quick eradication, and this policy is not likely to change. Better not have the disease than try to live with it is the thinking of veterinarians and livestock industry leaders. Over the past, this has been a wise course.

From the laboratory, results are already coming forth. A new low-cost method for producing foot and mouth disease virus for experimental studies and vaccination investigations has been developed. Kidneys from one calf supply enough cells now to prepare about 50,000 cultures in tubes and more than 300 large-scale cultures in quart-size flasks. The development of this tissue culture technique greatly reduces the number of test animals needed to produce the required supply of laboratory viruses essential to the research program.

According to information supplied, 400 steers as experimental animals were sacrificed last year. Next year, about 600 are expected to be needed. On the Island are 3,200 guinea pigs and about 5,000 mice, all being raised there to avoid the disease problem often encountered when these experimental animals are bought.

Other animals observed on hand at Plum Island were sheep, hogs, horses, rabbits, and chickens. Beef breed steers about 800 lb. in weight are the cattle most in demand for research studies. These steers are supplied from a disease-free source in Virginia.

Project International in Scope

Some people regard the animal disease research at Plum Island as wholly a United States project, but it was emphasized that it is really international in scope. Already, the results of scientific research from abroad have been incorporated into the laboratory's design and construction. Some of the top research men went to Europe to learn what was being done there. The knowledge gained from England, the Netherlands, and other countries has been very helpful in the establishment of the project here. It is expected that these inter-continental relationships will continue with the results of research passing freely from one country to another for the common good of the livestock industries and mankind.

Secretary Benson said in his address at the dedication ceremony, "Because scientists in other countries gave our specialists cooperation and permission to study in their laboratories, we are further along in our own research than we otherwise would have been. We appreciate their help. We will reciprocate. Knowledge is one good thing that all men share without in any way lessening their own store of it."

This laboratory definitely is a symbol of our will to advance and a mark of our determination to keep our farm livestock protected and healthy. The staff at Plum Island does not accept its responsibilities lightly. The dairy and livestock interests of this country will be well served as these scientists attack the monstrous tasks assigned.

As Old Fort Terry stood guard at the entrance to Long Island Sound and New York, so the Plum Island Laboratory on the same site stands alerted to protect the fifteen billion dollar livestock industry of this country against those "fifth column" animal diseases always waiting to sneak in. As the Secretary aptly pointed out, these would-be invaders are by far more menacing than the enemy fleets of 1898.

Antibiotics in Milk from Farms

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When the mammary glands of dairy cows are treated with antibiotic preparations in an effort to control or eliminate infections, the milk from such udders will contain objectionable quantities of these drugs for several milkings *(1, 2, 3, 10, 14)*. The amounts present, of course, will vary with the concentration and kind of antibiotic introduced, the carrier or vehicle employed in the preparation, the amount of milk drawn from the gland, and the time interval between treatment and milking *(1, 3)*. The milk may appear normal in every respect to the producer but the small amounts of antibiotic or antibiotics present, even when diluted with milk from untreated cows, are very often sufficient to cause trouble in the dairy plant receiving the milk *(3)*. They may also be sufficient to cause undesirable effects in milk consumers who are particularly sensitive to these drugs *(14)*. Furthermore, suspicions are being aroused that products made from milk containing antibiotics may contain infectious organisms that are antibiotic resistant and if implanted in human tissues are difficult for physicians to treat *(6, 12)*.

The Dairy Plant Problem

Just as antibiotics will destroy or inactivate some of the more common organisms causing mastitic infections in the udders of cows, so will they also destroy or inactivate most of the organisms used for developing acidity, flavor and curd character in cultured buttermilk, cultured cream, and a wide variety of cheese *(1, 3)*. The quality of all these products is dependent on the activity and normal functioning of the bacterial cultures used in the manufacturing processes. If the cultures do not act normally, it is either impossible to make the product at all or the product finally obtained is of poor quality and variable character *(13)*. Such inferior dairy products, if salable, bring a lower price and certainly affect consumption adversely.

Many dairies and cheese plants during the last 8 to 10 years have had more difficulty than was formerly experienced in manufacturing cultured dairy products because of the failure of cultures to ripen milk. Doubtless antibiotics in the milk supply have not been the cause of all the starter difficulties and failures experienced in this period, but there is little question but that they have been responsible for a significant proportion.

The only process used in the making of cultured dairy products and cheeses that might be expected to inactivate or destroy antibiotics in milk is that of pasteurization. Several investigators *(4, 7, 8, 9)* have shown, however, that the antibiotics commonly used for mastitis treatment, when in milk, are not significantly affected by the usual pasteurization heat treatments. Temperatures over boiling do reduce activities from 30 to 75%, but such temperatures also tend to caramelize the milk, alter its curd qualities, and render it unfit for the products in question.

Public Health Hazard

The medical literature has reported many cases of acute sensitivity to antibiotics among humans. Some people can not tolerate even very small quantities of these drugs whether they be injected by syringe or ingested by way of food. The levels of antibiotics sometimes found in milk are definitely dangerous to them.

It is becoming increasingly evident that the indiscriminate use of antibiotics by humans is dangerous. Schneierson, Mt. Sinai Hospital, New York *(11)* states that the use of antibiotics for trivial ailments may gradually build up a sensitivity, even in individuals who show great tolerance initially, thereby ruling out their use at a later time when a serious infection might make their employment vitally important. He further states that it is now well recognized that the residual effects of continued consumption of, or treatment with, antibiotics upsets the normal bacterial flora relationships of the body. It permits antibiotic-resistant organisms, previously harmless, to multiply, flourish, acquire virulence, and establish themselves as invaders. This often permits normally innocent molds to attack the mouth, rectum, vagina, and lungs, and the new infections are usually much harder to treat than the infection which invited the use of an antibiotic in the first place.

Another angle to the public health aspect of antibiotic residues in milk is that dairy foods made from such milk may contain unusual antibiotic-resistant strains of infectious bacteria capable of causing illness in humans. This suspicion was voiced in a Canadian note recently published in the Journal of the American Medical Association *(6)*. It was stated that when micrococci and streptococci, resistant to antibiotics, are encountered in patients with no history of hospitalization or antibiotic therapy, the cause may be found in the consumption of foods that contain such antibiotic-resistant bacteria. In support of this idea the findings of Thatcher and Simon were mentioned. These workers *(12)* examined 100 specimens of cheese obtained from the Canadian retail market. Sixty-seven per cent of the streptococci isolated and 15% of the micrococci were

found to be resistant to penicillin. The study revealed that the organisms isolated were most often resistant to penicillin and dihydrostreptomycin, the two drugs most frequently used in treating cows for mastitis.

The Federal Food and Drug Administration, in a statement of policy issued February 18, 1953 *(5)*, recognized the health hazard of antibiotics in foods and the possibility that their consumption may cause sensitization of consumers and may also result in the emergence of strains of pathogenic organisms resistant to antibiotics. The F.D.A. statement asserts that the direct or indirect addition of such drugs to foods may be deemed an adulteration under the food laws. The presence of antibiotics in milk coming from the farm is therefore illegal and is classified as an adulteration.

Prevalence of Antibiotics in Milk

Increasing amounts of antibiotics are being used in the treatment of mastitis, dosages are larger, and many milk producers fail to use the necessary care in keeping the milk from treated udders off the market until the antibiotic disappears. This requires at least six milkings for the more commonly used drugs *(14)*. There is also the possibility that antibiotics may be directly added to milk in an effort to reduce the number of viable bacteria. That the problem is becoming more acute seems evident from an F.D.A. survey completed early in 1955. This indicated that 11.6% of 474 samples of milk, collected nation-wide, contained measurable amounts of penicillin *(14)*. A survey made a year earlier found only 3.2% of 94 samples positive. It might be emphasized that of the 474 samples in the latest survey only 22 were raw milk and of these only one contained an antibiotic. This might be taken to indicate that the vendors of raw milk are much more careful with the milk leaving the farm than are those who ship to pasteurizing plants.

Threat to the Dairy Industry

The promiscuous and ill-considered use of antibiotics in treating mastitis in dairy cows, which seems on the increase, coupled with carelessness in keeping milk from such cows off the market, may be looked upon as a serious threat to the dairy industry. First, because antibiotics in the milk supply make high quality cultured dairy products impossible of attainment and may eventually seriously affect consumption of these products. Second, because the increasing incidence and concentration of antibiotics in milk and dairy products may create a health hazard which will become more generally recognized. If this should happen, milk and dairy products might receive a blast of unfavorable comment and criticism that would undo all the favorable publicity on food value that has been built up over the years.

The time seems ripe for dairy producers, dealers, and manufacturers to take a long, hard look at the antibiotic problem with the idea of reversing present trends. Unless this is done, there is an increasing likelihood that the industry may be put in an embarrassing and damaging position in the eyes of the public.

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Mastitis — The Struggle for Understanding

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In 1954 Reid published the results and interpretations of mastitis workers in Pennsylvania. Coming after years of work, this article seriously presented a concept thought to be correct (*Hoard's Dairyman,* **99**: 941. 1954).

Also in the northeast, New York and Connecticut conduct formal, organized mastitis control programs, which have been in operation for ten or more years. These two are the only such programs in the United States, and, although the concepts of mastitis overlap in important aspects, they still differ to a significant degree. Both concepts were built upon many years of experience, but both were contradicted extensively by the article mentioned above.

At the annual meeting held at Storrs, Connecticut, in 1956 the American Dairy Science Association committee on animal diseases reported that mastitis is the most costly dairy cattle disease not under satisfactory control. A representative of each of three states mentioned above was asked to present his views at a forum, the purpose of which must have been to achieve an understanding of the problem. Yet how can people in general hope to understand when there is little or no understanding among the men spending their lives on the problem?

The American Dairy Science Association has correctly concluded that mastitis is the most costly dairy cattle disease not under satisfactory control. It follows from this that some action should be taken to institute control where none now exists. But what action should be taken? Which of the three states has the proper concept on which to build? Surely such differences must ring warning bells in the heads of intelligent people. Something is wrong when, after all these years of trying to understand the problem, all we have are several concepts that appear to clash with each other.

An analysis of the situation yields only three broad possibilities: *(a)* mastitis is so amorphous that it can not be reduced to a single concept, *(b)* one of the concepts presented by the three states mentioned (or some other not mentioned) represents the proper concept, or *(c)* all the concepts fall short of the mark because the problem is larger than any single effort so far put forth toward its understanding.

As one who has devoted his entire career to the problem of mastitis the author contends that the third possibility is the correct one. Furthermore, it seems that the problem can be reduced to a simple outline by which a general understanding can be achieved. The following outline is proposed. It is arranged in eight points. Each succeeding point is dependent on those going before it. In using such an outline it is of the utmost importance to achieve a state of objectivity. The outline and our own experience must be studied in a detached, impersonal way, and understanding should be our objective.

Point 1

Much confusion arises from the many terms and classifications used in mastitis. For example, it is often stated that 20 or more kinds of infection have been found to cause mastitis. However, the truth of the matter is that only four of them account for at least 99% of all mastitis. These are streptococcal mastitis due to *Streptococcus agalactiae;* streptococcal mastitis due to other streptococci; staphylococcal mastitis; and bacillary mastitis (including coliform, *Pseudomonas,* etc.). These four are really separate diseases but, since the term mastitis is often used to include all forms of the disease they can be referred to either as forms of mastitis or as particular diseases.

Each of these forms of mastitis can occur in three degrees of severity. There is a nonclinical stage, in which an animal is negative to barn tests; a mild-clinical stage, in which an animal is positive to barn tests; and a severe-clinical stage, in which there is also swelling of the gland and possibly general illness of the cow.

Both of these deliberate consolidations are presented in the first part of the table, with the additional notation of YES under each of the 12 combinations. This is the first fundamental point: each of the four forms of the disease can appear in each of the three clinical stages. For example, bacillary mastitis can appear as nonclinical bacillary mastitis, as mild-clinical bacillary mastitis, and as severe-clinical bacillary mastitis.

Point 2

Recognition of the three clinical stages is within the ability of most people once they have had a little experience. Of course there will be overlapping due to variations in the care used in observing the signs of disease, but, generally, the three clinical stages will not be confused. However, it is only by the use of laboratory testing (culture) of milk samples that the different forms of the disease can be separated, and only by culture can an animal in a nonclinical stage be distinguished from a disease-free individual.

Thus the second fundamental point is that the clinical stages of each form of the disease can not be distinguished from each other without laboratory testing. For example, a case of mild clinical mastitis could be the result of infection with *S. agalactiae,* other streptococci, staphylococci, or bacillary bacteria, but no one

running the barn tests could tell which one was causing the trouble.

Point 3

For reasons that have never been adequately explained, the three clinical stages do not occur with the same frequency with each of the four forms of the disease. For example, *S. agalactiae* mastitis can be nonclinical or mild-clinical but only rarely becomes severe-clinical, whereas bacillary mastitis very often is severe-clinical. This third fundamental point is illustrated in the second part of the table by replacing the YES entries with + marks. The distribution of the + marks in the table is a generalization from the examination of many herds, and, as such, may not represent the actual situation in any one herd at any particular time.

Point 4

Within each form of the disease, the clinical appearance of the disease varies from time to time and from herd to herd. This fundamental point is represented in the second part of the table by ⟷ marks between clinical stages to denote the possible change of the disease back and forth from one clinical stage to another.

At one extreme it is possible, in any given herd, to have practically all infected animals showing mild-clinical or severe-clinical mastitis. At the other extreme, it is possible for a given herd to harbor all forms of the disease but to have them all in the nonclinical stage. Changes toward one extreme or the other can take place within days or weeks in the same herd, leading to what is often called flareups of mastitis. The precise reasons for such changes are not known.

Point 5

The different forms of the disease have different shedding characteristics. These are expressed in the third part of the table according to the duration of the infection itself and according to whether one can expect cultures to be positive when samples are taken at infrequent intervals. For example, the *S. agalactiae* form of the disease is more or less permanent, and cultures made at almost any time are usually positive, whereas in the bacillary form cultures made during the disease are often negative, and it is not uncommon for the disease to disappear completely in a matter of weeks. It is particularly to be kept in mind that the more severe the clinical signs, the more chance there is of getting a negative culture at that time.

Point 6

The *S. agalactiae* form of mastitis, one of the four main diseases of the udder, differs from the other three in the fact that its habitat is limited to the udder. It exists in the environment only a short time. If all udder infections are eliminated, the organism disappears from the environment. This simple fact has been known for years.

In addition, it has been established that the *S. agalactiae* form is peculiarly vulnerable to properly applied antibiotics such as penicillin. Apparently this vulnerability is not just a simple sensitivity to penicillin but involves the ease with which the bacteria are reached by the drug. Most *S. agalactiae* infections are curable, but in eradication any incurable cows must be removed from the herd.

Although these two facts have not been exploited to any great extent in this country, they make the disease eradicable from herds. Eradication is followed by improvement in both the quantity and the quality of the milk produced. It should always be remembered, however, that the eradication of *S. agalactiae* mastitis is the eradication of just one of four main forms of the disease. On the other hand, in spite of many statements to the contrary, there is as yet no acceptable evidence that the eradication of the *S. agalactiae* form leads to a greater incidence of the other forms.

Point 7

Unlike the *S. agalactiae* form of the disease, whose habitat is the udder of the cow, the habitat of the bacteria involved in the other forms of the disease is the environment. These other forms are found in many herds and often cause a great deal of clinical mastitis. Their mode of operation is still unknown, and the complexity of the problem has led some to the false conclusion that they are not actual diseases. However, in the absence of the knowledge we need they will remain uncontrollable in the specific, precise sense in which we can control the *S. agalactiae* form. In some herds and areas where the *S. agalactiae* form of the disease has been greatly reduced or eliminated, these other forms naturally receive greater attention and may constitute a serious, and very frustrating, problem. The knowledge needed for their control will be obtained only from research. At present there is practically no scientific effort being spent in this area, and it will require many dollars to obtain the needed facts.

Point 8

Since the clinical stages of mastitis are relatively easy to observe, in contrast to the difficulties involved in detecting the forms of the disease based on laboratory culture, it is not surprising that when most people talk about mastitis they are talking about the mild-clinical and severe-clinical stages of the disease. They do not realize that, while about one-half of all cows are not diseased at all, many are diseased but are in the nonclinical stages. Thus they erroneously conclude that, if cows are not showing mild or severe signs, they are disease-free, and they do not fully realize the frequency with which shifting can occur between the non-

clinical, the mild-clinical, and the severe-clinical stages.

An overwhelming majority of the modern treatments applied by farmers and veterinarians are administered solely on the basis of the presence of mild-clinical or severe-clinical mastitis. A true cure, based on adequate laboratory examination, is rarely determined, and the success or failure of the treatment is judged on the disappearance of the clinical signs of disease. Since the four forms of the disease can not be distinguished by the clinical signs, it is necessary to include in the treatments various drugs thought to be effective against each of the forms. Often treatments which are thought to be successful when the clinical signs subside have served only to force the disease into the nonclinical stage from which it can, and often does, flare up at some future time.

With so many farmers and veterinarians observing only the clinical aspects in the barn, and with so many different management and environment combinations to be observed, it is not surprising that a complex group of diseases can come to be connected in our minds with almost every conceivable factor of management or environment. Two of the three states mentioned earlier stress at least a dozen such factors, ranging from mud and dust in the barnyard, through the construction of the stalls, the feeding and milking of the cows, to the ventilation of the barn. Nearly everyone seems to overlook the fact that mastitis exists, and can be a serious problem, even in herds where the management and environment meet sensible standards.

The reasonable conclusion from this is really quite simple: The shifting between the clinical stages is the result of a battle going on between the infection in the udder and the cow. Thus two things must be considered: the ability of the infecting bacteria to injure the udder and the udder's ability to withstand injury. Naturally, many things happen as a result of management and environment that might upset the balance between the infection and the cow. Treatment is known to be able to tip the balance in favor of the cow, even when the causative infection is not cured. Often deliberate changes in the management and the environment aimed at mastitis control are followed by a reduction in the incidence of mild-clinical mastitis and severe-clinical mastitis; the disease is reduced, at least temporarily, to the nonclinical stage. So far, however, there is little or no evidence from controlled experiment to support or reject any of the dozen or more factors, and they must be considered on a trial-and-error basis.

The Laboratory Muddle

Three rather clear lines of action crystallize from the 8-point outline. However, the biggest obstacle to the general understanding of mastitis is the fact that there is no standard method for the laboratory culturing of milk samples. Probably not even two states use exactly the same method, although one state does operate six laboratories on a method standardized within their own organization. Without a standard test, many lines of thinking arise which are based not on differences in the thing being tested, but rather on the twists and side issues raised by the tests themselves. Under such conditions, even good tests can produce much misunderstanding.

Very little can be done without standardization, but who is going to bring about such standardization? So far nothing has been done by any source with authority enough to see the matter through. The Northeastern Mastitis Council, an unofficial group of mastitis workers in the northeastern states, has wrestled with the problem for the last five years, but without real success. It is not difficult to predict that success will not be achieved until some powerful, coordinating group accepts the responsibility.

It is not within the scope of this discussion to delve deeply into the matter, but a brief analysis of this situation should help many to understand the muddle in which we find ourselves. A method of laboratory culturing suitable for widespread use should possess three characteristics: It should be one that can be performed without undue difficulty; it should be one that can serve for the diagnosis of all forms of the disease in control work; and it should be one that with little or no modification can be used in research.

At this time it is only the second of these three characteristics (serving for the diagnosis of all forms of the disease) about which we need to be concerned in order to arrive at a preliminary judgment of the method in use in your particular area, and which, therefore, has served as the basis for the understanding existing in each area. Laboratory culturing begins in one of three ways:

(A) Refrigerating the unaltered milk samples until they are cultured in or on a solid culture medium (blood agar) which does not contain chemicals inhibitory to bacteria;

(B) Incubating the unaltered milk samples themselves prior to further examination such as by the use of the microscope or solid culture media; and

(C) Incubating the milk samples with the addition, either to the milk sample or to subsequent culture media or to both, of chemicals deliberately intended to prevent or subdue the growth of "unwanted" bacteria.

Only a method incorporating the procedure involved in *A* can be used for diagnosis of all forms of the disease regardless of which clinical stage they happen to be in. Methods incorporating the procedure set forth in *B* excessively magnify all the bacteria present in the milk sample, contaminating as well as infecting, and lead toward the erroneous conclusion that most

cows are infected. (The true situation is that only about one-half of all cows are infected, and practically all clinical mastitis is caused by infection.) Methods incorporating the procedure set forth in *C* are most numerous and were designed primarily for the detection of *S. agalactiae*. They magnify the presence of *S. agalactiae* while largely preventing the growth of other bacteria, including most of the organisms causing the other three forms of the disease. There are many variations of the *C*-type, and some (but by no means all) are capable of performing the specific job of detecting *S. agalactiae*. However, none of them can serve for the diagnosis of all forms of the disease regardless of which clinical stage the disease is in.

Thus a *B*-type of test shows too much false "infection" and leads to a distortion of the true situation. A *C*-type of test fails to show how widespread is infection with the forms of mastitis other than that due to *S. agalactiae*. Only on the basis of an *A*-type of test can the broad picture shown in the table be obtained. Once a satisfactory standardization of laboratory culturing is achieved, then, by means of an outline such as the 8-point one presented here, we should be able to speak the same language. It is then that three broad lines of action will appear.

First Action

The line of action suggested by point 8 comes first, not because it is most important but because it is the area into which most of our effort now falls. Most of our effort is in this area now because it involves the clinical signs which all can see. Our effort falls here also because proper laboratory culturing is not widely practiced and because drugs now exist by means of which we can attack this area.

When from a barn examination we say that a cow has mastitis, we mean that we see the signs of mild- or severe-clinical mastitis. When a drug manufacturer includes in his mastitis remedy several antibiotics and sulfas, he is doing so, even if he does not realize it, for the simple reason that we are trying to treat the clinical stages of up to four diseases without being able to tell them apart. When a long list of management and environment errors are mentioned to farmers as the cause of mastitis, it is because each one is believed to be related on some farms to either an increase or a decrease in the amount of clinical mastitis.

From the table, and from statements already made, the utter futility of thinking that mastitis can be controlled by the treatment of clinical mastitis only should be obvious. This is merely cutting the tops off the weeds and leaving the roots. On the other hand, it is also perfectly obvious that treatment helps hold the line. However, after about ten years of applying treatments in this way and considering that treatments are about as good as they are going to get, should we not give serious attention to the conclusion of the American Dairy Science Association that mastitis is the most important disease of dairy cattle which is not under satisfactory control?

Such an attack on the widespread use of treatment, and the futility of thinking that treatment alone can ever control the disease, usually is followed by a bombardment with many or all of the management and environment errors. The inference is that, although treatment alone will not control mastitis, the farmer can reach this desired goal if he will correct his errors. Few scientists working on mastitis will deny that there must be something of significance in this inference. However, there is probably less real information in this area of mastitis than in any other, and it would be difficult or impossible to find existing, adequate, scientific proof of the mastitis-causing effect of any of these so-called errors. It is obvious that good management would be good for cows, but the question is what is good management from the standpoint of mastitis? The problem is one of getting into operation, just as soon as possible, properly conducted and scientifically based research into the effects of various management and environment errors, while treatment holds the line.

Second Action

The second line of action is suggested by point 6: The *S. agalactiae* form of the disease can be eradicated from herds. Given the proper conditions, it is usually not difficult to eradicate, and its eradication is followed by a significant reduction in mild-clinical mastitis and a significant increase in milk volume. This action is the only precise, scientifically based, mastitis control action that it is within our power to take at present. Proper laboratory culturing of milk is absolutely necessary.

In general, there are three types of misunderstanding of this form of mastitis, and they should be easily corrected. The first stems largely from the use of *B*-type culturing, in which it is very easy to lose sight of the importance of the *S. agalactiae* form of the disease in the great mass of positive cultures shown by such methods. Unsound methods can lead only to unsound conclusions, and the simple correction is to change to an *A*-type method.

A second misunderstanding arises from the relatively mild nature of the *S. agalactiae* form of the disease. Since it is often found on farms in a nonclinical stage and at certain times on some farms may be entirely nonclinical, there is a tendency for people whose knowledge of the disease is superficial to question whether it really is a disease. However, the existence, nature, and importance of this form of mastitis have been adequately established over the years, and only in the face of very strong evi-

OUTLINE FOR THE UNDERSTANDING OF MASTITIS

Disease forms based on laboratory cultures	Clinical stages based on barn observations: Non-clinical Negative to barn tests*	Mild-clinical Positive to barn tests only	Severe-clinical Also swelling or general illness
POINT 1. Each of the four forms of the disease can appear in each of the clinical stages.			
POINT 2. Without laboratory cultures the clinical stages of each form cannot be distinguished from one another.			
Streptococcal, *S. agalactiae*	Yes	Yes	Yes
Streptococcal, other	Yes	Yes	Yes
Staphylococcal	Yes	Yes	Yes
Bacillary	Yes	Yes	Yes
POINT 3. The clinical stages do not occur with the same frequency in each form of the disease.			
POINT 4. All forms of the disease may fluctuate between the clinical stages, except that severe-clinical mastitis due to *Streptococcus agalactiae* rarely occurs.			
Streptococcal, *S. agalactiae*	+++ ⟷	++ ⟷	Rare
Streptococcal, other	+++ ⟷	+ ⟷	+
Staphylococcal	++ ⟷	++ ⟷	+
Bacillary	+ ⟷	++ ⟷	++
POINT 5. The four forms of the disease have different shedding characteristics.			
POINT 6. The *Streptococcus agalactiae* form of the disease is the only one that can be eliminated from herds. This action can be economically worthwhile.			
Streptococcal, *S. agalactiae* Long duration positive most days	+++ ⟷	++ ⟷	Rare
Streptococcal, other Variable duration positive most days	+++ ⟷	+ ⟷	+
Staphylococcal Variable duration not positive every day	++ ⟷	++ ⟷	+
Bacillary Short duration often negative when cultured	+ ⟷	++ ⟷	++

POINT 7. The habitat of these bacteria is the environment. It will be a monumental research task to discover their mode of operation. Until then they cannot be eliminated from herds.

*Barn tests such as strip-cup, bromthymol-blue test, Whiteside test, and the California mastitis test (CMT).

POINT 8. By means of treatment and management the clinical stages may be cured, or forced temporarily into the nonclinical stage. At present it is not known precisely which management practices are of true value.

dence should anyone allow himself to think otherwise.

A third, and perhaps most common, misunderstanding tends to minimize the importance of the *S. agalactiae* form of the disease because this organism is not found as the cause of severe-clinical mastitis nearly as often as are staphylococci and bacilli. A thorough study of the outline should make it obvious that an examination of only the severe-clinical mastitis cases of all four forms of the disease is no way to judge the occurrence and true importance of any one of the four diseases. This can be done only by studying all the cows in a herd, using as a basis a suitable *A*-type of culturing method.

Third Action

The third action, suggested by point 7, lies entirely in the field of research. So little is known about the mode of operation of the three forms of the disease other than that due to *S. agalactiae* that it is usually foolish to impose any control measures unless they cost little or nothing to impose, or unless they are imposed under very carefully controlled conditions so that some knowledge will be obtained.

Since the bacteria involved are apparently part of the environment, some investigators maintain that we will never be able to achieve a satisfactory degree of control over them. However, even though we know very little about the other three forms, we know enough to make it virtually certain that they are specific diseases for which specific counteractions could be devised. We have hardly begun to study them by scientific methods and, although it will be a difficult and costly job, it is high time that the work is started.

Conclusion

There are many people in responsible positions today who believe that mastitis can be controlled and that we know enough right now to satisfactorily handle this number one disease of dairy cattle. These same people, quite naturally, feel that research on mastitis is not needed nearly as urgently as is research on some of ar other diseases of dairy cattle. It is to be hoped that these people will study the outline for the understanding of mastitis and will see that only in one of the four diseases which comprise mastitis is there sufficient knowledge on which to base a precise, sensible, general control effort. With the other diseases comprising mastitis, information is lacking. Until we make the tremendous effort needed to correct this deficiency, the control of these other forms will remain in the empirical, trial-and-error area supported at best by testimonials.

Biochemical Aspects of Cheese Ripening

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The cheese ripening process results from the changes that occur during the storage of cheese that alter it from a bland, hard, rubbery mass to a smooth-bodied and full-flavored product. Cheese ripening is a complex system that involves numerous chemical, physical, and bacteriological changes, which are controlled by altering several environmental factors.

The history of cheese ripening is obscured in antiquity, but for each given cheese variety the curing practices have been handed down from generation to generation. Each cheesemaker knows how to treat his product to obtain a recognized cheese of the variety he desires, although he often is puzzled by the unwanted changes that frequently occur.

Type of Microorganisms Important

The ultimate quality of any given cheese depends upon both careful manufacture and proper ripening. The manufacturing procedure determines the future of the cheese by establishing the proper physical and chemical conditions under which ripening will proceed. The type of microorganisms, either in the milk or starter or added to the cheese; manufacturing methods; the general curing room practices; and the curing temperature and humidity are variable factors that combine to determine the cheese variety and the quality of the cheese within its recognized variety. The variable factors are altered for each specific variety, and often only minor variations differentiate cheese varieties. However, there are general concepts of cheese ripening that are applicable for all varieties. These fundamental concepts will provide a background for later discussion of the interrelationships that exist between cheese making and ripening and will show how the different variable factors involved in ripening are related to each specific variety.

Generally, the cheese ripening process is considered to begin when the cheese is placed in the curing room. However, in the broader sense, cheese ripening actually may be considered to start at the time the milk is drawn from the cow. From the time of milking until the milk is in the cheese vat, a bacterial flora becomes established in the milk. In raw milk cheese all of the organisms originally present may contribute to the ripening process, whereas in pasteurized milk cheese only those surviving the heat treatment will affect the ripening. Milk with an extremely low bacterial count is not always suited for cheesemaking since flavor development in cheese is frequently dependent on the activity of naturally present organisms, even in pasteurized milk cheese. To date, insufficient knowledge of the specific roles of the various microorganisms has prevented the use of bacteria-free milk. On the other hand, improperly handled milk usually contains too many bacteria of the wrong species to make good cheese. Even after pasteurization such milk rarely makes high quality cheese since an improper flora often has initiated undesirable fermentations which may continue into the cheese ripening process.

The bacterial starter cultures are essential to provide in the green curd the physical and chemical properties that will favor desirable changes during ripening. Also, the starter organisms may contribute more directly than is generally realized to the development of desired flavor in the cheese.

Rennet Plays an Important Part

Rennet, which is used in the manufacture of most types of cheese, has as a primary function the coagulation of the casein, but its proteolytic activity continues during the ripening process. Except for the types of rennet con-

taining lipases, such as rennet paste, it is difficult to conceive that rennet has a direct bearing on development of characteristic cheese flavor. Today rennet extract generally is used, which means that the same enzyme(s) is added to the milk regardless of type of cheese desired.

Primary and Secondary Changes

The changes taking place during cheese ripening may be divided into two general parts. The first part, termed primary changes, includes changes that occur in carbohydrate, fat, and protein, which result in the accumulation of lactic acid, fatty acids, and free amino acids (the primary compounds). The second part, termed secondary changes, involves the formation of compounds brought about by action of enzymes primarily from microorganisms on the primary compounds. The relative rate and the pathways by which both primary and secondary changes proceed determine the type and quality of cheese. However, it is difficult to draw a distinct line where one terminates and the other begins.

The primary changes in the carbohydrate are initiated by the starter organisms, lactic acid–producing bacteria added to the milk to create proper acidic conditions in the cheese. Starter organisms along with rennet extract also contribute to the primary changes in protein. The primary changes in fat are generally brought about by milk lipases or animal lipases added to the milk with the rennet.

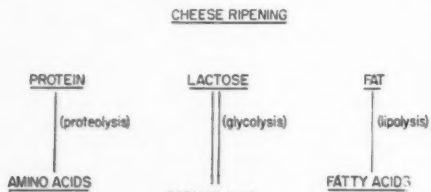

FIG. 1. "Primary" biochemical changes during cheese ripening.

Figure 1 indicates the conversion of lactose, protein, and fat to primary degradation products. These primary steps are often the entire description given to cheese ripening. The conversion of lactose to lactic acid during and after manufacturing is essential in all cheese varieties. Glycolysis, as this process is termed, requires about 14 different enzymatic steps. The lactic acid formed plays an important part in the character of the cheese and the subsequent ripening. Cheese varieties vary as to when the major part of the lactic acid is produced in the manufacturing process. Varieties such as Cheddar and Provolone require almost complete acid development before the manufacturing process is completed. In other varieties, such as Brick, the acid is developed mostly on the drainage table and sometimes is not completed until after the cheese is placed in the brine tank. Regardless of the stage at which lactic acid is formed, most of the lactose originally present has disappeared after 24 hours with only trace amounts of glucose and galactose detectable for the next 7 to 14 days. Galactose generally may be detected for a longer period than glucose.

Based on the results of various studies conducted at the Ohio State University during the past 7 years, Figure 2 shows products of lactose

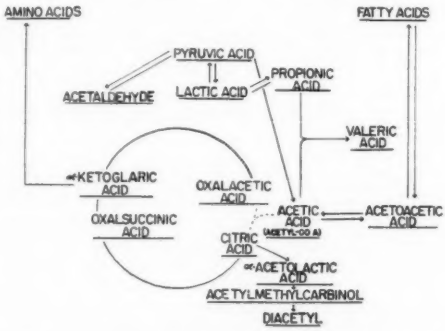

FIG. 2. Metabolic pathways and products of lactose and citrate fermentation in cheese.

fermentation isolated from one or more cheese varieties. All of these products are well known metabolic products, but most of them can be characterized as intermediate — which means that they are between the beginning and the end of the fermentation. Each variety of cheese has its own chemical pattern consisting of certain intermediate metabolic products. The difference may be either quantitative or qualitative, depending on how closely related the varieties are to one another. Cheese deviating from desired variety characteristics usually shows variation in the intermediate metabolic pattern in proportion to the deviation.

The lactic acid bacteria added for the purpose of development of acidity begin to decrease in numbers shortly after the cheese has been made. At the same time the microorganisms responsible for the secondary changes begin to become more dominant. Some of these microorganisms were present in the milk, but others are often added to the cheese somewhere in, or just subsequent to, the manufacturing process. However, continuation of primary changes, particularly liberation of amino acids and some fat hydrolysis, is brought about by these organisms.

Probably the best known example of an important secondary reaction in cheese involving lactic acid is its conversion to propionic acid in Swiss cheese. The role of lactic acid with

regard to formation of characteristic flavor in other types of cheese is not too well understood at the present time.

Fat hydrolysis occurs to some extent in all cheese varieties but is more important in some than in others. In cheese such as Blue and Romano the action of lipase has been shown to be essential to the development of characteristic flavor.

Secondary changes in fat which have been demonstrated in cheese of various varieties are shown in Figure 3. Some of the compounds are

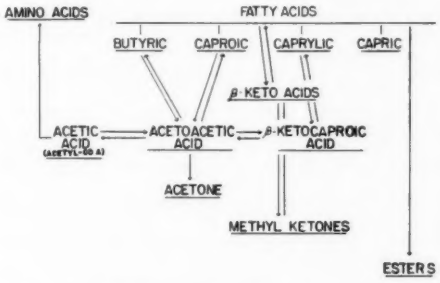

FIG. 3. Metabolic pathways and products of fatty acid catabolism in cheese.

intermediate compounds in fat metabolism; others may be considered end products. Both primary and secondary degradation products of fat are powerful flavor and aroma components of cheese. The degree to which the different components contribute is roughly inversely proportional to their molecular weight.

Protein degradation varies with the variety of cheese. Primary protein hydrolysis to amino acids occurs to some extent in all cheese varieties. In certain types, such as Limburger and Camembert, proteolysis is much more extensive than in types such as Swiss. Figure 4 demonstrates secondary changes in protein degradation products of amino acids. Again all of these products have been demonstrated in one or more cheese varieties. It is recognized that amino acids contribute to the background flavor of cheese, but whether amino acids add to a characteristic flavor is problematic. Perhaps proline is a specific part of Swiss flavor and glutamic acid of

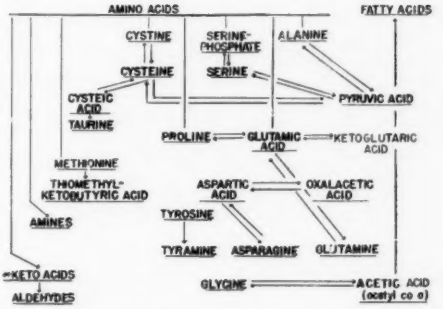

FIG. 4. Metabolic pathways and products of amino acid catabolism in cheese.

Provolone, but in most varieties amino acids probably just serve as a background flavor with certain ones being utilized as building blocks for bacterial protein and others as sources of energy.

In the degradation process certain gases are released, NH_3, H_2S, and CO_2. Ammonia and H_2S contribute directly to cheese flavor, whereas CO_2 contributes indirectly.

The actual pathways and subsequent accumulation products involved in each cheese are varied and depend on the combination of enzyme systems that are present and active in the cheese system. Some of the degradation products classified as secondary in this article actually are of a tertiary or quaternary nature. Perhaps some microorganisms or enzyme systems operative in cheese should be classified as tertiary, etc., according to the type of degradation product they utilize. That successive enzyme systems or bacterial cycles exist in cheese was demonstrated with a Romano cheese held for a prolonged period of time. The cheese reached a peak in flavor and then slowly deteriorated. Accumulation of butyric acid, acetoacetic acid, acetaldehyde, and formaldehyde were demonstrated in that order, indicating a stepwise degradation of fat involving at least four different enzyme systems.

This presentation of changes taking place in cheese during ripening has been general in its scope. Specific changes as they occur in each variety of cheese will be discussed in subsequent articles.

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* Abstract of paper presented at annual meeting.
*e Abstract of paper presented at Eastern Division meeting.
*w Abstract of paper presented at Western Division meeting.

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